Interactions Between
Streptococcus mutans and *Veillonella dispar*

by
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Professors Michael Wilson, Brian Henderson and Robert Seymour

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Declaration

I Jeen Will Koning confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Furthermore all microarray data will be released in a publicly available database in line with MIAME standards.

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Abstract

Dental plaque, a collection of bacteria forming a biofilm, is the cause of the most common diseases of mankind: caries and periodontal diseases. This study reports on the interactions occurring between two key species in this biofilm *Streptococcus mutans* and *Veillonella dispar*. These organisms are hypothesised to form a cooperative metabolic system in which a waste product, lactic acid, produced by *S. mutans* is utilised by *V. dispar*. The objective of this thesis was to evaluate whether these species cooperate to determine whether knowledge of their interaction can be applied to prevent caries.

The hypothesis was examined in single and dual species planktonic cultures and biofilms which were continued for up to 14 days, with their growth, vitality, microstructure and environment closely monitored. *S. mutans* gene expression was quantified during two stages of biofilm growth to determine the effects of *V. dispar* co-culture. A qualitative model was developed that coupled the growth and decline of *S. mutans* and *V. dispar* around lactic acid. Experiments were conducted *in silico* to determine whether the interaction could be modulated to improve health, and the model was expanded to include a third species to investigate the production of lactic acid as a competitive strategy.

The results demonstrated the two species had a mutually beneficial relationship initially, but lactic acid rapidly accumulated and killed *V. dispar*. *S. mutans* gene expression changed considerably in co-culture, including upregulation of bacteriocins. The initial hypothesis that the species cooperate because *V. dispar* removes lactic acid was rejected as *S. mutans* produces lactic acid as a chemical warfare agent and does not want it removed.

The principal conclusion is that *S. mutans* employs a strategy of acidogenicity and aciduricity to gain dominance in the dental plaque biofilm. This strategy overwhelms the benefit from cooperative interactions that remove lactic acid and thus sociobiological approaches to prevent caries should focus on competitive interventions.

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
ºC	Degrees Celsius
cDNA	Complementary Deoxyribonucleic Acid
cfu	Colony Forming Units
CO_2	Carbon Dioxide
COGs	Clusters of Orthologous Groups
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EPS	Exopolysaccharide
g	Acceleration due to gravity
GCF	Gingival Crevicular Fluid
h	Hour
H_2O_2	Hydrogen Peroxide
kb	Kilobase
kV	Kilovolts
L	Litre
Log	Logarithm
µg	Microgram
µg ml^{-1}	Microgram Per Millilitre
µl	Microlitre
min	Minute
mg	Milligram
ml	Millilitre
mM	Micro-Molar
N	Population Size
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
r	Growth Rate, Malthusian Parameter
RNase	Ribonuclease
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per Minute
s	Second

tRNA	Transfer Ribonucleic Acid
TSB	Tryptone Soya Broth
TSBA	Tryptone Soya Broth + Agar (solid but not Tryptone Soya Agar as recipe different)

1 Introduction

> *"The most important unanswered question in evolutionary biology, and more generally in the social sciences, is how cooperative behaviour evolved and can be maintained in human or other animal groups and societies"*

That is how Lord Professor Robert May began his last presidential address to the Royal Society on the 30th of November 2005, and this thesis reports on an approach taken to address this question, not by studying animal groups or even Eukarya, but by investigating cooperation in the far more numerous and genetically rich Domain, Bacteria. In bacteria, hypotheses originally posed for how cooperative behaviour can be maintained in animal societies, can be more rigorously studied. Bacteria exist in nature as complex and dynamic communities (Hall-Stoodley et al. 2004). These complex communities comprise many different species of bacteria that compete and cooperate with each other. In understanding how bacteria interact, new methods to control them may be identified. The specific interactions that occur are difficult to identify amongst the multitude of interactions that occur between the many species in a bacterial community (Cadotte et al. 2005). This necessitates breaking the community down in to manageable but meaningful pieces to identify interactions. This study investigates the interactions occurring between two key bacterial species in dental plaque that play different roles in the induction of caries. It focuses on how the species grow in the presence of each other, and whether there are differences in growth rates, maximum population sizes and survival. This study also investigates whether the presence of the other species affects the environment they each can live in and how they change their environment. These two species are investigated using both experimental and mathematical methods. This study also takes advantage of the power of genetic analysis to investigate if one bacterial species changes the genes it expresses in the presence of another. Consequently this introduction gives an overview of the types of social interactions that can occur between any types of organisms. It then discusses bacteria, bacterial growth, microbial ecology, biofilms, and how bacteria interact with each other and their environment. Penultimately it focuses in on the two species under investigation and their role in the formation of dental caries before concluding with a discussion on how the use of genetic analyses can help unravel the interactions that occur between species, and how mathematical methods are employed to understand biological phenomena.

1.1 Social Interactions: Competition, Cooperation, Altruism and Spite

This thesis sets out to add to the understanding of the evolution of social behaviours including cooperation in microorganisms to better discern the underlying principles of altruistic cooperation, whose explanation is one of the greatest challenges in evolutionary biology. Current understanding of how animals and plants behave can be applied to bacteria as the behaviours of plants, animals and bacteria have all been shaped by evolution. Equally, knowledge gained about bacterial behaviours can be transcribed back to animals and plants, with the stipulation that bacteria do not reproduce sexually and consequently bacterial populations can have very high relatedness. The interactions that occur between organisms can include competitive, cooperative, altruistic and spiteful behaviours and the ensuing relationships may be considered as parasitic (where one organism benefits at the expense of another), mutualistic (where both organisms benefit), commensal (where one organism benefits while the other is unaffected) or competitive (where both organisms are harmed). All organisms have evolved to behave in a manner that maximizes the transmission of their genes. They grow and reproduce, although sometimes they do not reproduce directly (for example worker bees help the queen bee and forego reproduction themselves and birds help their family members raise chicks) but in these cases the individuals behaving altruistically are still maximizing their inclusive fitness because they are related to the young they are helping to raise. Bacteria reproduce through binary fission so they form clonal populations of almost genetically identical individuals. This high relatedness makes it evolutionary advantageous for bacteria to behave cooperatively with clone mates as they are helping almost identical copies of themselves.

Kin selection is very good at explaining cooperation among relatives and in 1964 Bill Hamilton formalized the relationship by stating that an organism should perform a costly action if the relatedness of the organism to the recipient multiplied by the benefit to the recipient is greater than the cost to the organism.

$$rb > c$$

This equation nicely explains worker bees helping to raise their queen's young and altruistic behaviour towards relatives. Since relatedness is one within a clonal population of bacteria, the r can be removed from the equation leaving kin selection theory stating that a bacterium should perform a costly action if the benefit to the recipient is greater than the cost. Kin in this context means organisms that have copies of the same genes, and relatedness is the proportion of the genes that are the same. A human mother and her daughter would have a relatedness of 0.5 as the daughter has 50% of her mothers genes while two bacteria in a clonal population have a relatedness of 1.0 as 100% (or very close to 100%) of their genes are identical. Moving from investigating selection acting on individuals to selection acting on genes changes r to the relatedness of the gene or genes controlling the behaviour under selection. Thus horizontal transmission of genes in bacteria can result in different species of bacteria having a relatedness of 1.0 for the transposable element if they share it and bacteria that have grown through binary fission having a relatedness of 0 for certain genes, if there has been a mutation or the acquisition of a transposable transposable element where one bacterium has it and the other doesn't. Kin selection nicely explains cooperation between related organisms, however, there are many types of behaviours, and normally these occur between unrelated organisms. Unrelated species permit an investigation of how cooperative behaviours can be maintained in the absence of kin selection.

Table 1.A Classification of behaviours between organisms.

		Effect on Recipient	
		+	-
Effect on Actor	+	Mutualistic	Selfish
	-	Altruistic	Spiteful

When an actor behaves in a way that is beneficial to itself but costly to another organism it is classed as a selfish (or parasitic) behaviour. Conversely, mutualism, altruism, selfishness and spite may all be cooperative behaviours (selfishness and spite if the actor is punishing the recipient for not behaving cooperatively) but typically cooperative behaviours have a positive effect on the recipient. Bacteria that show growth restraint, where individuals employ metabolic pathways that maximise growth yield over growth rate would be considered as behaving mutualistically as all

parties benefit. The production of siderophores (iron chelating compounds) which help bacteria scavenge iron from the environment is an altruistic act as the bacterium that produces them has no guarantee that it will benefit from them as it has to release them out into the environment for them to bind to iron (West and Buckling 2003). A bacterium that relies on other bacteria to produce siderophores but does not produce them itself is behaving selfishly, as it benefits while other bacteria pay the cost. The production of bacteriocins (toxic compounds) is a spiteful behaviour as they are costly to produce and toxic to others. All of these behaviours are selfish as the bacteria expect to benefit at some level but the three that are not classed as 'selfish' can all be cooperative behaviours.

However with bacterial biofilms, different bacterial species live together as communities and bacteria within these communities perform behaviours that are beneficial (cooperative behaviours) to individuals from other species. This can not be explained by kin selection as the individuals are unrelated (or rather their closest ancestor may have been millions of years previously). Why help members of another species given that all behaviours are inherently selfish? Explanations include shared interest, to reward partners who cooperate, to punish partners who do not cooperate (sanctions, policing) and indirect reciprocity (West et al. 2003).

1.2 Bacteria

Bacteria are unicellular microorganisms. They are prokaryotes and lack cell nuclei or any membrane bound organelles, along with the Archaea (which differ genetically and structurally). Figure 1.A shows a diagram of the structure of two major groupings of bacteria, Gram-positive and Gram-negative bacteria. These groupings mainly differ in their cell wall structure with Gram-positive bacteria having a thick peptidoglycan layer (sugar and amino acid mesh that forms a cell wall) while Gram-negative bacteria only have a thin peptidoglycan layer but have an extra outer membrane containing lipopolysaccharide (a covalently bound lipid and polysaccharide that are important for the structural integrity of the bacterium but elicit a strong immune response in animals).

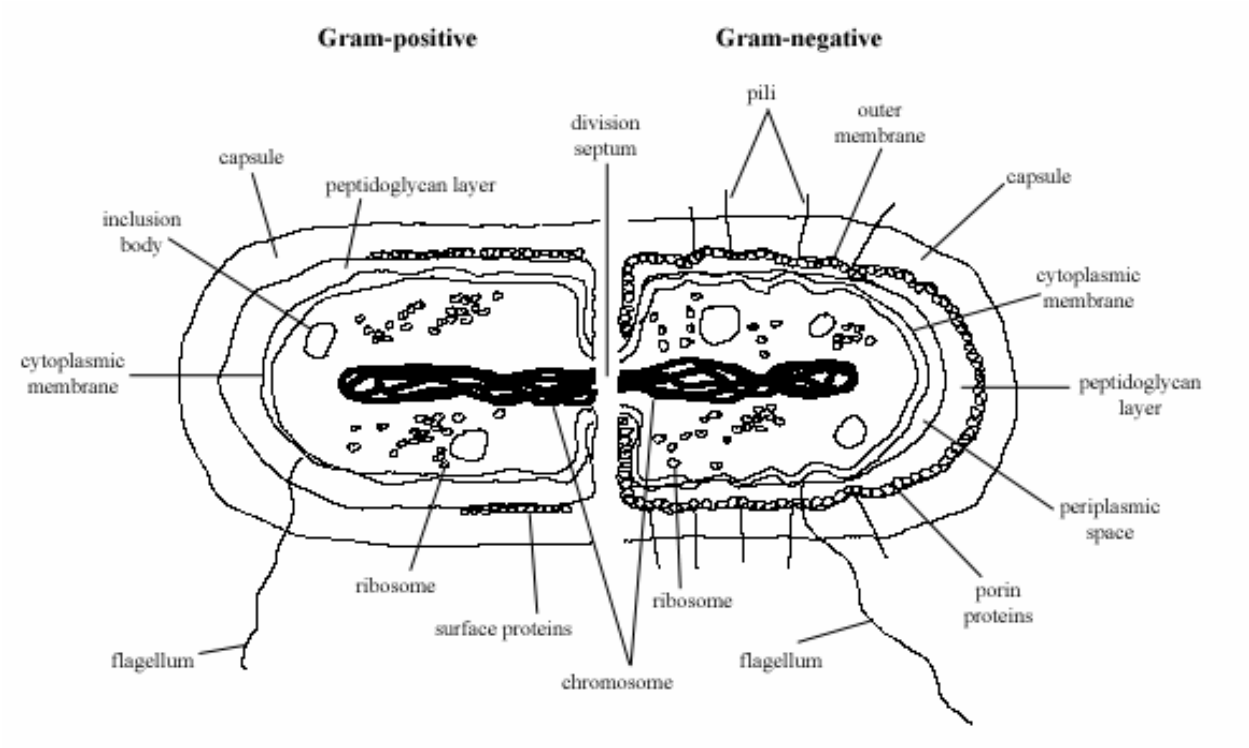


Figure 1.A Diagram of the structure of Gram-positive and Gram-negative bacteria This figure shows the key components of the bacterial cell for the two major groupings of bacteria, Gram-positive bacteria which retain crystal violet when gram stained due to their thick peptidoglycan layer and Gram-negative bacteria which do not retain crystal violet due to their much thinner peptidoglycan layer and are thus stained by a counterstain (normally safranin which is pink). This image is redrawn and updated from http://wikieducator.org/Bacterial_Structure and it is reproduced in a modified form under the creative commons license.

There are 4-6 x 10^{30} bacteria living in all habitats on earth (Whitman et al. 1998) and possibly on other planets (Dartnell et al. 2007, Horneck 2008). There are 10 times as many bacteria in our human microbiota as there are of our own cells (Savage 1977, Gill et al. 2006). Very few species of bacteria have even been identified and of these very few are pathogenic, but, not surprisingly, these are the ones that are well studied. Bacteria are hugely important in many ecosystem processes, particularly for the cycling of nutrients, either as nitrogen fixers or through the decomposition of organic matter. Three things are key for bacterial fitness; growth, survival and dispersal.

1.3 Bacterial Growth

Bacteria grow exponentially through a process of fission. This growth occurs at a characteristic rate when the bacteria are provided with suitable nutrients and physical conditions. This growth rate is called r or the Malthusian parameter (Charlesworth 1970, Velicer and Lenski 1999). When nutrients start to run out or toxic waste products interfere with growth, the rate of growth ceases to be exponential and slows until growth stops altogether.

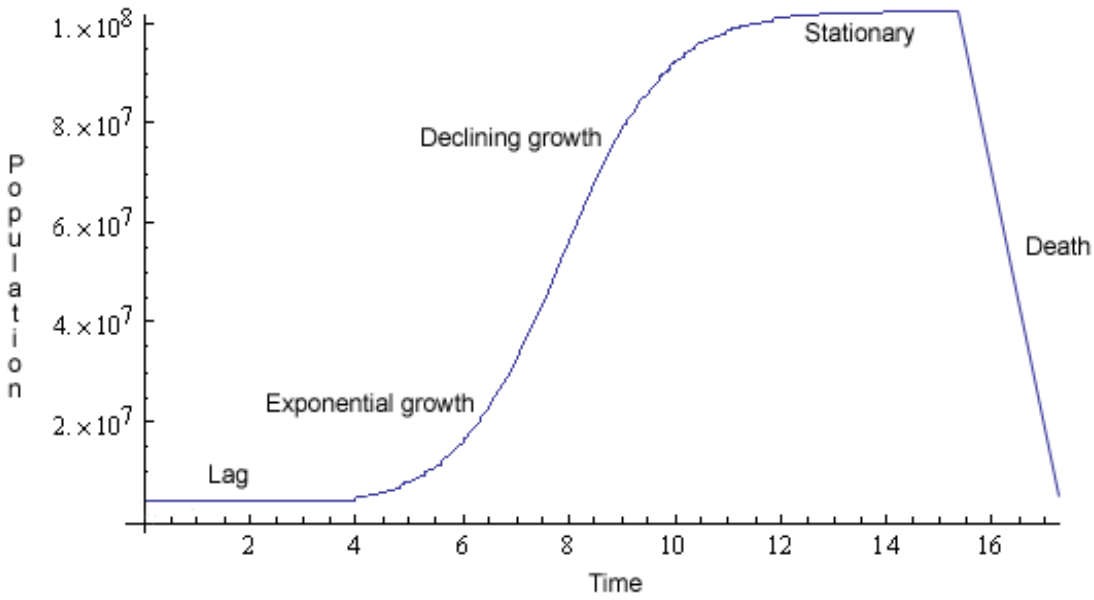


Figure 1.B Diagram of five stages of bacterial growth.

There are five distinct phases of the growth and decline of bacterial populations: lag, exponential, declining growth, stationary and death (Beckers and van der Hoeven 1982, Christian et al. 1982, see Figure 1.B). The lag phase is when bacteria inoculated in to a medium do not begin to grow immediately. The length of the lag phase and whether it even occurs depends on a variety of factors including the organism, the growth phase of the inoculum, and the new medium in to which they have been introduced. During this time, the bacteria undergo physiological changes adapting to their new conditions. In the exponential phase the bacteria grow through a process of fission at a constant rate (r). The declining growth phase is when bacteria are still growing but the rate of growth is decreasing due to nutrient scarcity or the accumulation of metabolic products with toxic actions. The stationary phase occurs when a nutrient becomes exhausted or a metabolic end product has accumulated to such a

concentration that it prevents growth. The death phase is when the bacteria die through starvation or damage due to toxic products in the environment. The exponential growth phase, the declining growth phase and the stationary phase can be described mathematically using the logistic growth equation, where t represents time, r represents the growth rate, and K represents the carrying capacity (maximum population size which occurs during the stationary phase) and N represents the actual population size at time t (Tsoularis and Wallace 2002).

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right)$$

This equation is one of the oldest examples of the application of mathematical modelling to biology and it is still one of the most useful.

Bacteria are usually studied planktonically (free-living and floating in liquid) *in vitro* as pure cultures. However, in natural situations bacteria do not usually grow as single cultures but exist with other bacteria usually in the form of a mixed species biofilm (Costerton et al. 1999). Biofilms can comprise only a single species but typically they comprise many species. Bacterial biofilms are communities of bacteria that form at phase boundaries and generate an extracellular matrix composed of polymers such as polysaccharides and nucleic acids (Sutherland 2001, Wimpenny 2000). This matrix provides adhesion, protection and facilitates social interaction (Wimpenny 2000). The properties of biofilms are very different to those of the individual bacteria of which they are comprised (Costerton et al. 1987). Biofilms tend to be less susceptible to antibiotics and other environmental stresses than planktonic bacteria as a consequence of the dense extracellular matrix that encases them and the rich assortment of proteins and chemicals it contains (Mah and O'Toole 2001, Mah et al. 2003). Bacteria that live as biofilms have markedly different phenotypes to planktonic bacteria and express different genes (Waite et al. 2005, Mikkelsen et al. 2007, Mikkelsen et al. 2009, and Shemesh et al. 2007).

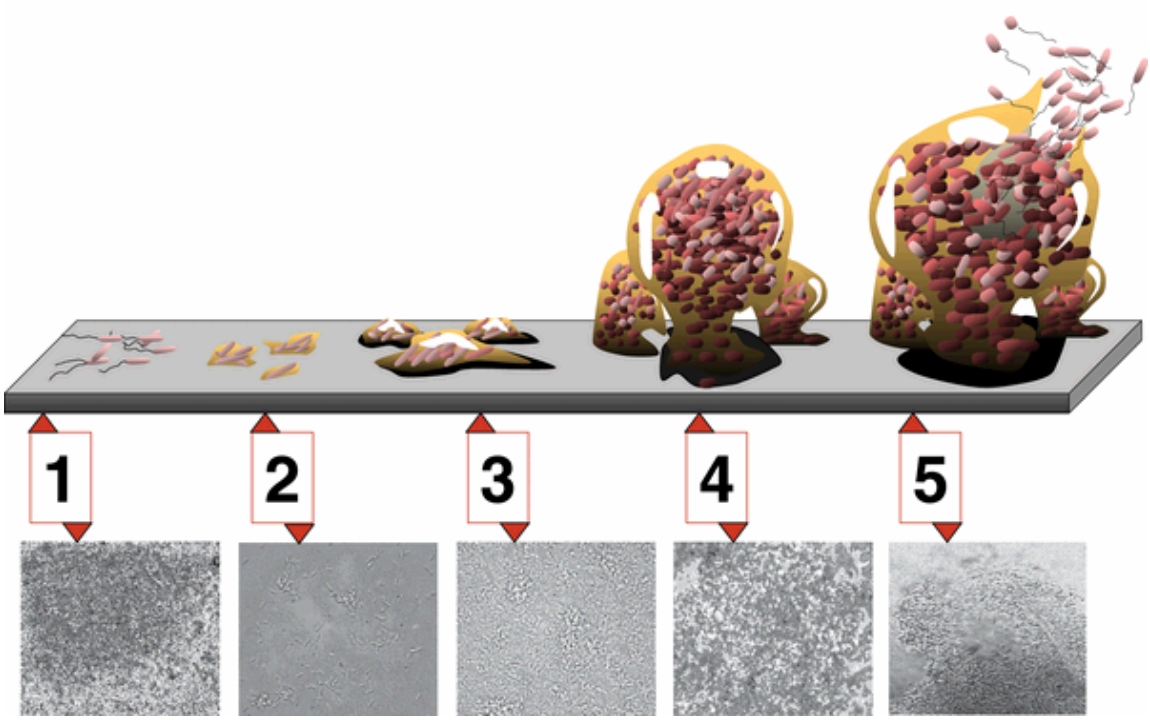


Figure 1.C Phases of biofilm growth.

Photomicrographs of *Pseudomonas aeruginosa* development (below) with diagrams of what the bacteria are doing during each phase (above). Biofilm stages of growth are attachment (stage 1), irreversible attachment (stage 2), maturation I (stage 3), maturation II (stage 4), and dispersal (stage 5). The next stage is of biofilm decline and is called the death phase. All photomicrographs are the same scale. This figure is reproduced from

http://www.plosbiology.org/article/slideshow.action?uri=info:doi/10.1371/journal.pbio.0050307

(Monroe 2007, Image credit: D. Davis) under the creative commons license.

Bacteria growing as biofilms go through phases of growth and decline similar to the five phases described above and these phases are: attachment, irreversible attachment, maturation I, maturation II, dispersal and death (See Figure 1.C, Kierek-Pearson and Karatan 2005, Monroe 2007). The maturation I phase is similar to the exponential and declining growth phases of planktonic bacterial growth but is not limited to fission as biofilms can also grow through recruitment. The maturation II phase is similar to the stationary phase but typically lasts for longer as bacteria in biofilms are more resilient than planktonic bacteria (Mah and O'Toole 2001).

Bacteria are influenced by and influence their environment. The rate of bacterial growth is dependent on temperature and pH and bacteria have minimum, optimum, and maximum temperatures and pH for growth. Bacteria that inhabit humans have

adapted to grow best at an optimum temperature of between 32 and 37ºC, the temperature of the surface and core, respectively, of the human body. Most bacteria grow best around neutral pH with limits between 5 and 9, but acidophilic bacteria can grow at low pH, and some bacteria are able to grow at extremely low pH, < 3 (Padan et al. 1981, Johnson 2008). Often when an environment has a low pH it is as a consequence of bacterial metabolism. Some species of acidophilic bacteria produce acid waste products from the metabolism of sugars thus lowering pH (Schachtele and Leung 1975, Patel et al. 2006).

1.4 Microbial Ecology

Bacteria have typically been studied in isolation. However natural bacterial populations are incredibly complex and microbiologists and biologists in general are increasingly studying systems rather than individual components. The study of species in isolation (axenic culture), and the reductionist approach have proved very successful but due to advances in technology and the vast amounts of data produced using the reductionist approach and the exponential increase in computing power, further advances in all fields of biology will result from using an integrative systems approach to put all the parts together and thus gaining an understanding of the whole that is more than the sum of its parts (Ideker et al. 2001, Weston and Hood 2004, Noble 2006, Sauer 2006).

Despite their small size and limited number of genes, bacteria are social; they live together in communities (Crespi 2001, Henke and Bassler 2004). This understanding has revolutionised the field of microbiology as it was originally assumed bacteria lived independent lives without displaying cooperative behaviours that have elicited so much interest in scientists studying animals. Bacteria communicate and cooperate to perform a variety of behaviours including dispersal (fruiting bodies, Branda et al. 2001), nutrient acquisition (siderophore production, West and Buckling 2003), ‘chemical warfare’ (bacteriocins, Gardner et al. 2004), quorum sensing (density dependent behaviours, Fuqua et al. 1994) and biofilm formation (where many of the other behaviours listed here often occur simultaneously, Costerton et al. 1999). These behaviours are interesting in their own right, in how they have evolved and because many of them are involved in bacterial virulence. Explaining cooperation is

one of the greatest problems for evolutionary theory. Bacteria are particularly useful for addressing this problem as behaviours are genetically controlled, and many bacterial genomes are fully sequenced (Jessup et al. 2004). Scientists can manipulate experimental conditions while quantifying gene expression to identify genetic mechanisms that underlie social traits. Key discoveries are being made about evolution (Rainey and Rainey 2003), altruism and selfishness (particularly regarding cheating; Travisano and Velicer 2004, Velicer 2003) and cooperation and conflict (Xavier and Foster 2007) using bacteria as model systems.

1.5 Biofilms

Where organisms behave in an altruistic or cooperative manner over a period of time complex societies, where individuals work together to achieve goals they could not achieve alone, may evolve. Biofilms are communities of microorganisms, encapsulated in an extracellular matrix of polymers, that live at interfaces. While biofilms may grow at liquid/liquid interfaces or liquid/gas interfaces, they are typically attached to a biotic or abiotic surface that interfaces with liquid or gas. Although when biofilms grow at a solid/gas interface, the immediate surface tends to be moist. Most, if not all bacteria, can form biofilms (Kolter and Greenberg 2006). Biofilms do not solely comprise bacteria and may include algae, archaea, fungi and protozoa. They may include all of these different types of organisms or in rare cases they may form a monospecies biofilm. The extracellular matrix is made up of varying combinations of polysaccharides, proteins and nucleic acids (Branda et al. 2006). Bacteria when living as biofilms are many times more resistant to antibiotics, biocides and hydrodynamic shear forces than when the same bacteria employ a planktonic phenotype (Schembri et al. 2002). Species richness and the extracellular matrix act as buffers against changing conditions, although when conditions do change the composition of the community and matrix change over time (Freeman and Lock 1995, Sutherland 2001, Kolter and Greenberg 2006). Figure 1.D shows two different dual species biofilms, one visualised using confocal microscopy and one using scanning electron microscopy.

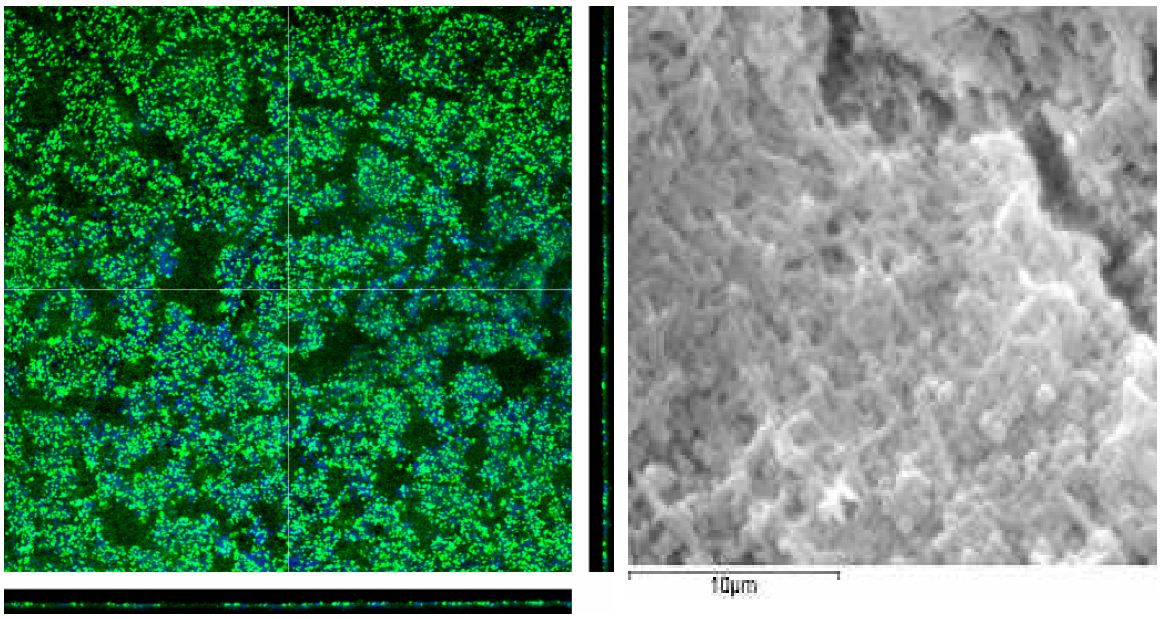


Figure 1.D Confocal micrograph and scanning electron micrograph of biofilms. The confocal micrograph (on left) shows a 300µm square dual-species biofilm after one day of growth, with live bacteria dyed green and dead bacteria dyed blue. The side projections (to the right and below the image on the left) of the confocal micrograph show vertical and horizontal slices through the biofilm. The Scanning electron micrograph shows the surface topology of a dual-species biofilm after one day of growth. A 10µm scale bar is shown below the scanning electrom micrograph.

1.6 Dental Plaque, Caries and Periodontitis

Humans are heavily populated with bacteria and the vast majority of these bacteria are harmless or even beneficial (Ruby et al. 2004, Wilson 2008). However some bacteria which normally coexist relatively benignly with humans may under certain conditions grow out of control, produce harmful waste products or both. The two most ubiquitous bacterially induced diseases of humans are tooth decay (caries) and gum disease (gingivitis or, more broadly, periodontitis) and these are caused by dental plaque (see Figure 1.E). Figure 1.E shows dental plaque on teeth stained with neutral red. Figure 1.F shows a scanning electron micrograph of dental plaque and Figure 1.G shows a diagram of a tooth with one side healthy and the other side showing signs of caries and periodontitis.

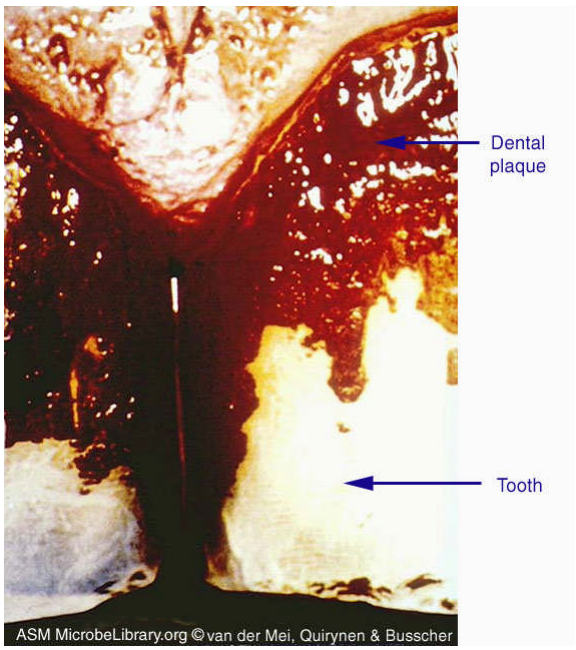


Figure 1.E Dental plaque biofilm *in situ* after nine days of no oral hygiene.

The biofilm which is normally colourless is stained with neutral red so it is visible. The image is reproduced with permission from www.microbelibrary.org. (Image copyright H. van der Mei, M. Quirynen and H. Busscher; original reference Wilkins 1999).

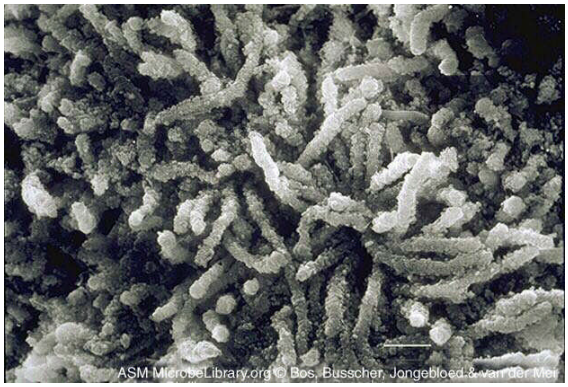


Figure 1.F Scanning electron micrograph of human dental plaque. In this figure coaggregating bacteria form corncob like structures. Each kernel of the corncob structure is a bacterium. The scale bar = 10 µm. The image is reproduced with permission from www.microbelibrary.org. (Image copyright: H. Busscher, H. van der Mei, W. Jongebloed, R. Bos).

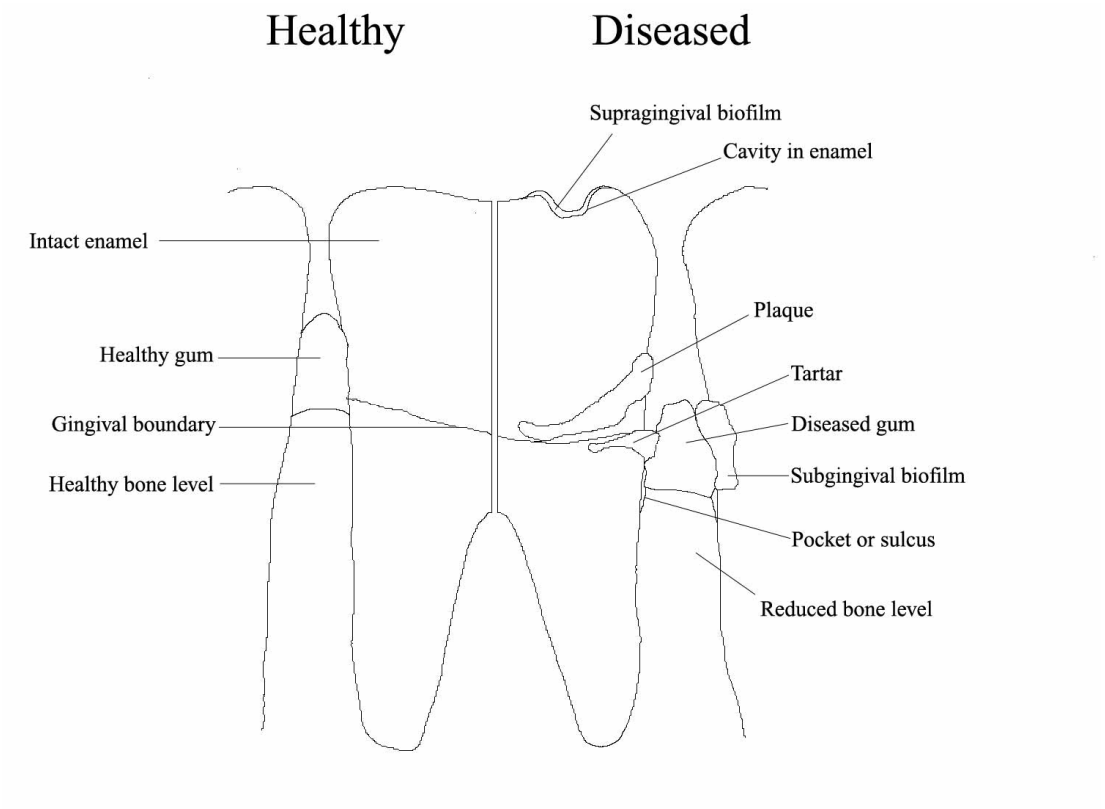


Figure 1.G Diagram of a tooth showing a healthy side and an unhealthy side with caries and periodontitis. Bacterial biofilms are shown in this figure as plaque and as tartar (calcinated plaque) and these biofilms can exist above (supragingival biofilm) or below (subgingival biofilm) the gingiva (the gums). Bone loss, decreased gum size, pockets between the gum and the tooth and a cavity are shown on the unhealthy side of the tooth.

The dental plaque biofilm is one of the most studied and understood biofilms in existence. Plaque is a biofilm consisting of bacteria, salivary polymers and bacterial extracellular products that adhere to the surface of the teeth (see later section 1.9). Most biofilms comprise many species. Massively parallel pyrosequencing has identified 6888 species level phylotypes in human plaque and 3621 in saliva which results in an estimate of 19000 phylotypes in the oral cavity (based upon the number of phylotypes identified given the number of samples taken and the number of ribosomal subunits sequenced, Keijser et al. 2008). This is the number of bacteria that were found in the oral cavity but it does not mean they are resident in the oral cavity, that is some of these bacteria will have been sampled even though they do not reside in the oral cavity but were just there by chance. The vast majority of these species are unnamed and unstudied and any effect they have on human health is unknown, although any negative effect is likely to be small, as harmful bacteria are

often identified as the damage they cause attracts attention (with the notable historical exception of *Helicobacter pylori* causing ulcers and gastritis, Warren and Marshall 1983, Marshall 2002). This diversity is due to the identification of many rare species. In a different study that considered prevalence in the oral cavity, 875 taxa could account for 99% of 34,753 clones sampled (Dewhirst et al. 2010). Metabolic products (acids) produced by a few members within this community may result in irreversible damage to tooth enamel in the condition known as dental caries. Dental caries affect 60-90% of school children and most adults in industrialized and developing countries (World Health Organisation, Petersen 2003). Treatment of dental caries is estimated to account for between 5-10% of the health-care budget of industrialized countries, making it the fourth most expensive disease and treatment is beyond the resources of many developing countries (World Health Organisation, Petersen 2003). Dental plaque biofilm is also responsible for periodontitis, inflammation of the soft tissues around the teeth that can lead to gum disease and bone loss (see Figure 1.G). Plaque that is not removed can become calcinated over time, turning into tartar (also called calculus) which is very difficult to remove (White 1997). Oral biofilms growing on the teeth differ dramatically, depending upon the local environmental conditions and the age of the biofilm, but they can be roughly divided in to supragingival biofilms (on exposed enamel) and subgingival biofilms (below the gum within the periodontal pocket) (Wilson 2005, Kolenbrander et al. 2010, see Figure 1.G). Bacteria that gain entry to the pulp or the gums can invade the capillaries and then be disseminated around the bloodstream, and some of these bacteria may cause diseases such as infective endocarditis (usually caused by viridans streptococci or *Staphlococcus* spp. binding to valves in the heart, Wilson et al. 2002). The factors involved in the development of caries are the composition of plaque bacteria, the structure and composition of enamel (i.e. fluoride content which can incorporate into enamel forming fluorapatite), the host's diet, the host's immune response and the composition and flow rates of saliva (Wilson 2005). The factors that counter the drop in pH and arrest the formation of caries are the depletion and then exhaustion of the carbohydrate supply, the removal of carbohydrates by saliva, the removal and buffering of acid by saliva, the host's immune response, the production of basic compounds by plaque bacteria (e.g. NH_3), the metabolism of lactic acid by plaque bacteria and the decreased metabolism of acidogenic bacteria due to low pH (Wilson 2005). There are a small number of bacteria implicated in the pathology of dental caries *comprising Streptococcus mutans*, *Streptococcus sobrinus* (together

called the mutans streptococci), *Lactobacillus* spp. and *Actinomyces naeslundii* (Wilson 2005). *S. mutans* is the primary causative agent of dental caries and it is described in the next section (Loesche 1986).

1.7 Streptococcus mutans

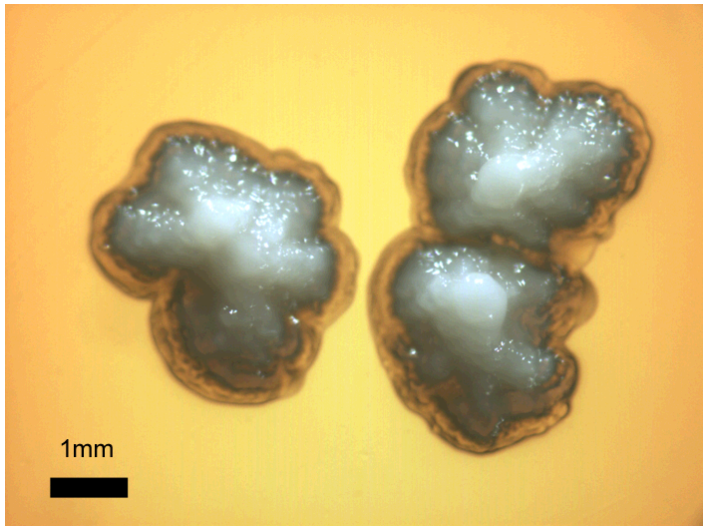


Figure 1.H Three *S. mutans* colonies grown on agar and viewed using a light microscope at 200 times magnification.

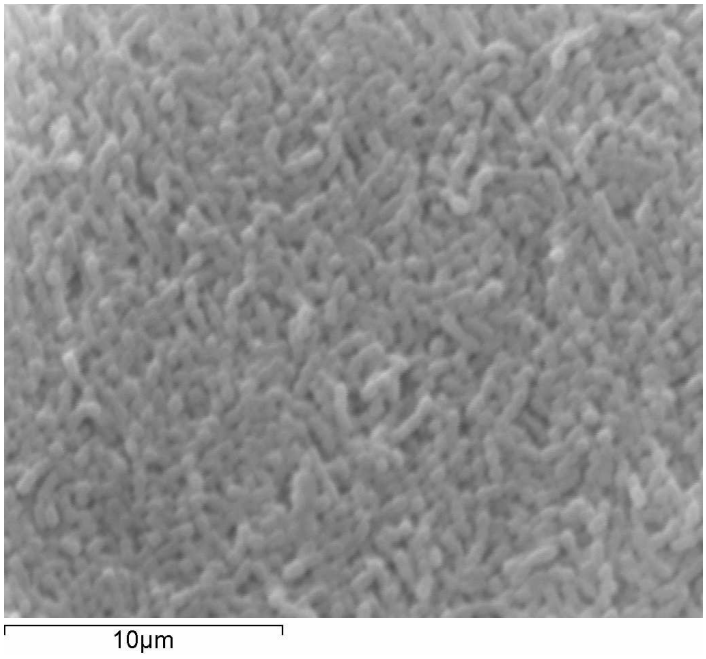


Figure 1.I Scanning electron micrograph of *S. mutans* biofilm

S. mutans is a Gram-positive bacterium, which plays a key role in the formation of the dental plaque biofilm as an early coloniser (it produces adhesins which attach the organism to the acquired pellicle of the teeth) and is the most important bacterium in the formation of dental caries (Clarke 1924, Loesche et al. 1975, Loesche 1986). The structure of *S. mutans* can be seen in the representative Gram-positive bacterium shown in Figure 1.A but note that *S. mutans* do not have flagella, but do have pili (Kline et al. 2010). Figure 1.H shows three colonies of *S. mutans* growing on agar. *S. mutans* are Gram-positive ovoid cocci, that typically occur in pairs or chains, are aciduric (grow well in acid medium) and acidogenic (produce acid) and are non-motile facultative anaerobes that grow optimally at 37°C (Wilson 2008). Figure 1.I shows pairs and chains of *S. mutans* tangled together in a dense biofilm. *S. mutans* are nutritionally fastidious; for anaerobic growth, a representative strain required the organic compounds: glucose, cysteine (or thioglycolic acid), biotin, p-aminobenzoic acid, thiamine, riboflavin, pyridoxine, pantothenic acid and nicotinic acid and for aerobic growth, uracil and one of the amino acids, asparagine, aspartic acid, glutamic acid or glutamine had to be included in the medium (Carlsson 1970). Except for the requirement of nicotinic acid, ammonia is sufficient as the sole source of nitrogen for growth (Carlsson 1970). They are incapable of respiratory metabolism and their primary energy supply is from the fermentation of carbohydrates into predominantly lactic acid (Wilson 2008). The loss of enamel from teeth is the result of lactic acid production by *S. mutans.* When lactic acid donates protons to hydroxyapatite, the main component of enamel, the hydroxyapatite breaks down producing calcium ions, phosphate ions and water (Margolis and Moreno 1992).

$$Ca_{10}(PO_4)_6(OH)_2(s) + 8H^+(aq) \rightarrow 10Ca^{2+}(aq) + 6HPO_4^{2-}(aq) + 2H_2O(l)$$

1.8 Veillonella dispar

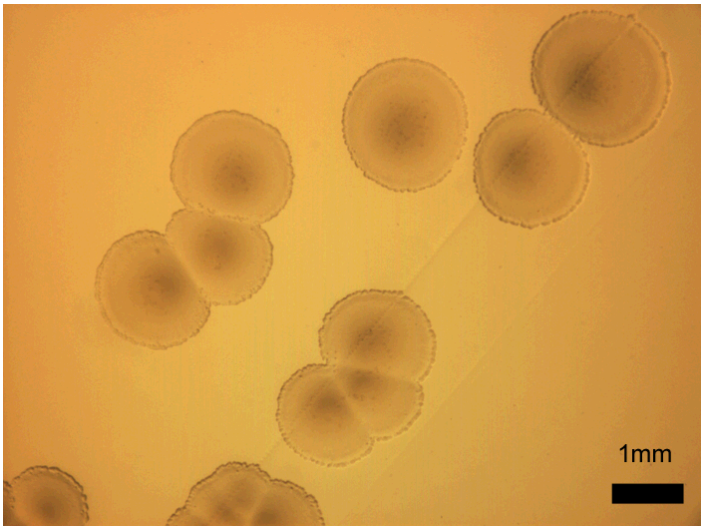


Figure 1.J Fifteen *V. dispar* colonies growing on agar and viewed using a light microscope at 200 times magnification.

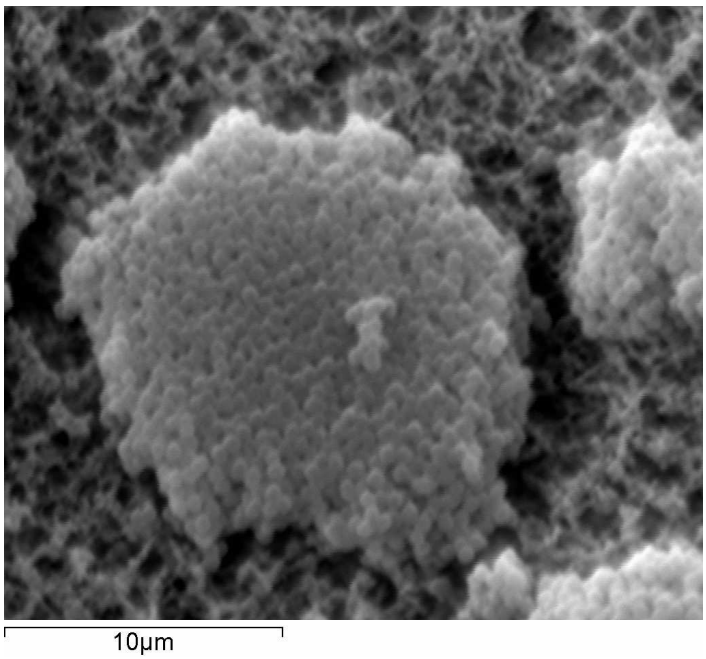


Figure 1.K Scanning electron micrograph of *V. dispar* biofilm

Veillonella dispar is a Gram-negative anaerobic bacterium, which as an early but not primary coloniser in the dental plaque biofilm plays a key role in the metabolism of lactate and is thus hypothesised to reduce the occurrence of caries (Rogosa 1965, Mays *et al*. 1982, Hoshino and Sato 1986, see Figures 1.J, 1.K and 1.L). The structure of *V. dispar* can be seen in the representative Gram-negative bacterium shown in Figure 1.A but note that *V. dispar* do not have flagellae. *V. dispar* are obligate anaerobes that usually occur in pairs or clusters and are non-motile cocci (Wilson

2008). Figure 1.K shows a scanning electron micrograph of a *V. dispar* biofilm where individual cocci are clearly visible. They are nutritionally fastidious and grow well with the organic compounds cysteine, thioglycolic acid, biotin, p-aminobenzoic acid, folic acid, thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, casein, hypoxanthine, putrescine, uracil, tryptophan, tyrosine, proline, phenylalanine, histidine and lactic acid (Rogosa and Bishop 1964), but while they benefit from lactic acid for growth, it is not required. *V. dispar* can not use carbohydrates as an energy source, and use fumarate, lactate, malate, pyruvate and some purines instead and generate acetate, propionate and hydrogen as metabolic waste products (Wilson 2008). This study focuses on these two species because of their role in the production and removal of lactate. This shared metabolic pathway may influence the occurrence of social interactions between these species and the literature widely states that these two species cooperate because they share this metabolic pathway (van der Hoeven et al. 1978, McBride and van der Hoeven 1981, Mikx et al. 1972, Mikx and van der Hoeven 1975, Kara et al. 2006, Palmer et al. 2006, Chalmers et al. 2008).

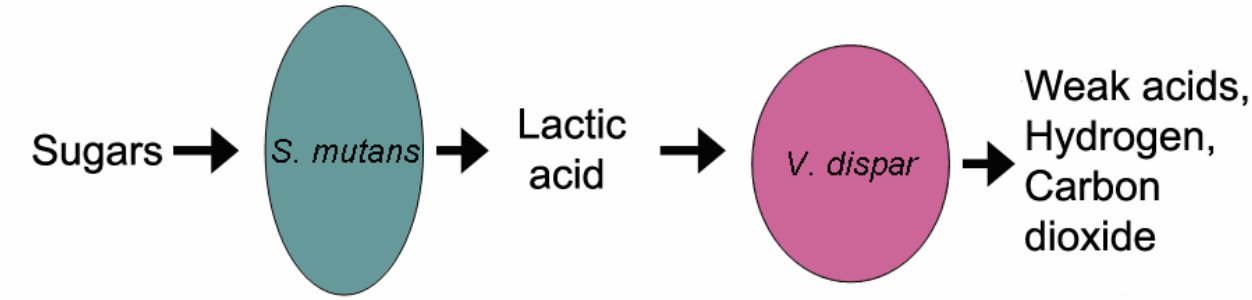


Figure 1.L Shared metabolic pathway of *S. mutans* and *V. dispar*. *S. mutans* metabolises glucose and other sugars into lactic acid which *V. dispar* then metabolises into weaker acids, carbon dioxide and hydrogen. The available carbohydrate and the population size of *S. mutans* limit the amount of lactic acid that can be produced, and the population size of *V. dispar* and the acidity of the environment limit the amount of lactic acid *V. dispar* can process (an acidic environment can place stress on *V. dispar* and limit its metabolism and a very acidic environment can kill *V. dispar*).

1.9 Development of the Dental Plaque Biofilm.

The development of dental plaque involves the colonisation of the tooth, subsequent development of microcolonies and coaggregation of additional bacteria, thus forming a complex and dynamic community, which under certain conditions can rapidly become pathogenic. The surface of the tooth is covered in a pellicle consisting mainly of proteins, lipids and glycolipids that act as a substratum for biofilm adhesion but also protect the enamel against demineralisation and aid in its remineralisation (Wilson 2008). Compounds present in saliva wash over the teeth and some bind to form the pellicle. Initial pioneering colonisers bind to specific molecules in the acquired enamel pellicle employing molecule specific adhesins (Wilson 2008). Pioneering species include *Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Actinomyces* spp., *Neisseria* spp. and *Haemophilus* spp. (Idone et al. 2003, Wilson 2008, Kolenbrander et al. 2010). Once the pioneering species have bound, other species then bind to them using adhesins as illustrated in Figure 1.M (Kolenbrander et al. 2010). *S. mutans* can bind directly to the pellicle and employs two methods to do so, sucrose dependent binding (water soluble and insoluble glucans) and sucrose independent binding (cell-surface proteins that function as adhesins), although, while *S. mutans* can bind to the pellicle itself, it normally joins the plaque biofilm as an early coloniser after the attachment of some of the pioneering species mentioned above (Lee et al. 1989, Kuramitsu 1993, Jenkinson 1994, Ajdić et al. 2002, Idone et al. 2003, Wilson 2008). *V. dispar* is an anaerobic species that joins the biofilm once it has developed sufficiently to provide anaerobic niches but it is still an early coloniser and present from the early stages of plaque development (see Figure 1.M, Kolenbrander et al. 2010). *S. mutans* has been added to a figure originally published in Nature Reviews Microbiology and this adapted figure is shown here (Figure 1.M, adapted from Kolenbrander et al. 2010). Connections (lines) have been added to the figure to show coadhesion and sucrose independent binding (Bradshaw et al. 1998, Ajdić et al. 2002, Ledder et al. 2008).

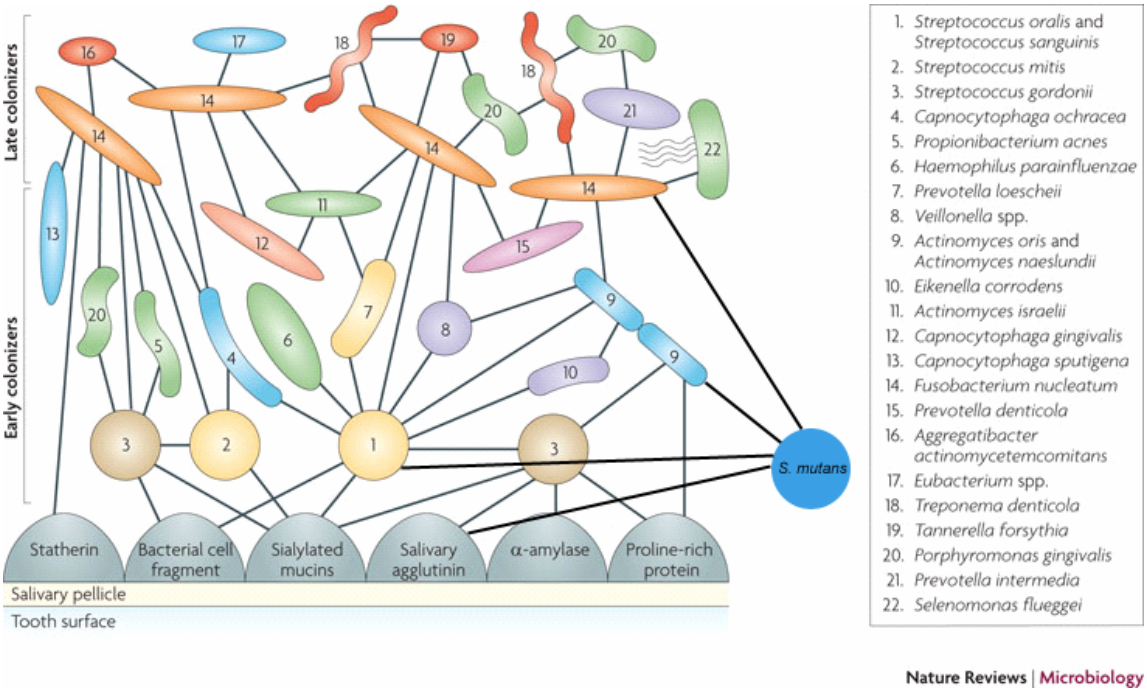


Figure 1.M Colonisation of the tooth and development of the dental plaque biofilm. Spatiotemporal model of colonisation of the salivary pellicle on the tooth by early colonisers and subsequent coaggregations between early colonizers, fusobacteria and late colonizers. These spatiotemporal attachments are fundamental in the development of dental plaque. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Kolenbrander et al. 2010), copyright (2010), which itself was adapted with permission from American Society for Microbiology: [Microbiology and Molecular Biology Reviews] (9), copyright (2002).

Bacteria join the biofilm through binding to the extracellular matrix or through binding to a bacterium in the matrix (coadhesion). Coadhesion is the binding of a planktonic microorganism to a microorganism immobilised on a surface, and/or in a biofilm while coaggregation is the binding of two planktonic microorganisms through the binding of components on their cell surfaces (Kolenbrander et al. 2010). Both processes involve adhesins on the surface of one cell binding to receptors on the surface of another cell and it is likely the mechanisms involved are identical (Rickard et al 2003, Kolenbrander et al. 2010). This permits assays to be conducted to determine which bacteria can bind to each other in biofilms (coadhesion) by vortexing planktonic solutions and observing whether the bacteria bind (coaggregation). When *S. mutans* and *V. dispar* were assayed in this way, only 2.8% of bacteria bound (Ledder et al. 2008), however both species bind strongly to *Fusobacterium nucleatum* (Bradshaw et al. 1998) indicating how complex multi-species communities can

come together to form biofilms held together by adhesins (and extrapolysaccharide matrix). *F. nucleatum* coaggregates with initial, early and late colonisers and is considered a bridge species in the development of dental plaque (Kolenbrander et al. 2010). As planktonic bacteria are washed away by saliva and swallowed before they can grow it is imperative for their survival in the oral cavity that they anchor themselves and it is proposed that the multitude of specific coaggregation pairings that occur determine how the successional plaque community develops (Kolenbrander et al. 2010).

The successional development of the plaque biofilm, which for the most part causes no damage to the host, can develop into a dynamic yet balanced microbial community (microbial homeostasis), akin to a climax community of a forest, where community composition may change but remains relatively rich (Kolenbrander et al. 2010). Dental plaque studies that have been conducted over several years have shown climax communities are remarkably stable, despite changes in environmental determinants resulting in shifts in proportions of members and occasional eliminations and introductions (Wilson 2008). This has led to the ecological plaque hypothesis which relates dental caries to an ecological catastrophe, where changes in metabolism lead to changes in the pH of the environment, which subsequently changes the composition of the community and this continues in a negative feedback loop until only a few acid tolerant species remain (Marsh 1994, Marsh 2003, Kolenbrander et al. 2010). Rather than the biofilm buffering the pH and restoring it to a more neutral pH through a range of mechanisms, the community of aciduric species persist in an acidic environment which destroys the enamel.

1.10 Dual Species Biofilms

Extensive studies have been conducted on dual-species batch cultures (where bacteria are grown in liquid medium), but few studies have investigated dual-species biofilm interactions (Komlos et al. 2005). Bacteria still interact and may coaggregate in liquid culture (Rickard et al. 2004) but planktonic growth rates do not necessarily predict dual species population densities (Komlos et al. 2005). Some studies have shown cooperation and beneficial interactions between bacteria growing as dual species biofilms (Skillman et al. 1999, Cowan et al. 2000) and others have shown

cooperation, competition and neutral interactions (Christensen et al. 2002, Simões et al. 2007). Christensen et al. (2002) showed that competitive or cooperative interactions could arise depending on relative spatial structuring within a dual species biofilm. In a similar dual species biofilm pairing to the one investigated in this study (between *S. mutans* and *Veillonella parvula*), Kara et al. (2006) found similar biofilm growth for both species in single and dual species biofilms but that dual species biofilms accumulated less lactic acid and were less susceptible to antimicrobial treatment (chlorohexidine). In another similar pairing, Gutiérrez de Ferro et al. (1999) prepared a medium that was suitable for *Streptococcus* species to grow, contained D-glucose, and contained every nutrient *Veillonella* species need to grow but no energy source for the *Veillonella*. They then demonstrated that a *Veillonella* clinical isolate could not grow on this medium by itself but that it could grow in the presence of a *Streptococcus* clinical isolate and thus concluded the *Veillonella* was using a waste product of *Streptococcus*, most likely lactic acid, as its energy source. Most interestingly, Hansen et al. (2007) demonstrated the evolution of a symbiotic relationship in a dual species biofilm (*Pseudomonas putida* and *Acinetobacter* sp. Strain C6), where simple mutations in the genome of one species caused it to adapt to the presence of the other, and this derived community was more productive than the original community.

1.11 Experimental Models

S. mutans is well studied and is the major cause of dental caries, and *V. dispar*, while not as well studied forms a metabolic relationship with *S. mutans* so the pair are useful both for the study of the evolution of cooperation and with the aim of identifying ways to modulate the system to reduce the incidence of caries. There are a number of different models used to study biofilm growth (e.g. Annular Reactor, CDC Biofilm Reactor, Constant Depth Film Fermentor [CDFF], Flow-cell [Flow-chamber], MBEC Assay™, Membrane filters, Microtiter plate assay, Modified Robbins device, Sorbarod filter [reviewed by Pratten and Ready 2010]) and these include techniques that employ continuous culture, batch culture or solid medium. They also employ different substrata on which the biofilm grows. Some of these methods include artificial shear forces, pulsing of nutrients, replenishment of nutrients and removal of waste. In this study, biofilms were grown on membrane filters

placed on agar, as this method allows large numbers of bacteria to grow, as it is a simple method to employ and because of the reasons outlined below. In contrast to many of the other biofilm growth models; nutrients are depleted, wastes accumulate, and the biofilm grows at an air/solid interface with nutrients diffusing through the nitrocellulose filter. The depletion of nutrients and accumulation of wastes are interesting features of this study as they cause the biofilm to go through all the stages of biofilm growth and decline. Additionally, the accumulation of wastes (e.g. lactic acid) is also relevant as lactic acid is nutritionally important to *V. dispar*. It is also interesting to investigate the effects of nutrients diffusing through the agar and growing the biofilms at an air/solid rather than liquid/solid interface.

1.12 Mathematical Models

Models are a simplified representation of a system, based on hypotheses and equations, used to rationalise observations (Picioreanu and Loosdrecht 2003). In general, a quantitative description is superior to a qualitative one as it describes the system in rigorous detail, and is useful for making predictions. A mathematical model is an attempt to translate the conceptual understanding of a system into mathematical terms and relationships (National Research Council 1990). If the mathematical model reproduces the relevant phenomena, it indicates the conceptual understanding of the system and the way it has been quantified are justified (Wanner et al. 2006). Modelling improves our conceptual awareness of systems by helping understand the fundamental mechanisms that are operating and the way they are linked. Modelling can be used to develop testable hypotheses, trial experimental design, create novel process designs and improve the performance of a process. Models may be simple or incredibly complex depending upon their purpose.

Systems biology is the systematic study of complex interactions in biological systems, where computer models attempt to put the biological components, be they chemicals, cells or organisms, together in such a way that the models capture the emergent properties of systems. These emergent properties are the properties of systems that can not be explained by summing the properties of the individual components. For example, biofilms have very different properties to those of individual bacteria. There are two main advantages of applying a systems biology

approach; firstly the correct recreation of a system identifies key components and the way they come together, and secondly, once a system is created *in silico* (in a computer rather than *in vivo* or *in vitro*) it can be subject to tests unavailable to the experimenter. This allows the rapid testing of a wide spectrum of parameters cheaply and without risk.

1.12.1 Mathematical Representations of Biological Systems

The models used by systems biology to recreate biological processes vary from the simple (e.g. BCT, Bray et al. 1993, a model of bacterial chemokinesis) to the very complex (e.g. the Heart Model, Noble 2006). The models may describe the behaviour of signalling molecules (Bhalla 2003), cells (Endy and Brent 2001), tissues (Yamada and Cukierman 2007), whole organs (Noble 2006), individuals (i.e. personalised medicine; Weston and Hood 2004), or populations (Brauer and Castillo-Chavez 2000).

Models can be deterministic where the system change is defined and includes no randomness and thus will always return the same result, or stochastic, where the system changes due to predictable processes combined with a random element. They can be single-scale where processes occur at the same time and spatial scales, or multi-scale where processes occur at multiple time and/or spatial scales. Models may be qualitative, comprising a set of numerical equations defined by constraints (e.g. physical and biological), or quantitative (as qualitative but including statistical methods). They may be simple or complex and may involve single or multiple species. They may be non-dimensional (not having a spatial component or having all physical units removed and being self-referential), or may be multi-dimensional (having multiple dimensions or aspects). The variables can be kept in a steady-state where all variables are constant despite processes occurring that would otherwise change them (steady state models) or the variables may be dependent upon time (dynamical models). Finally, they may model a single process or multiple processes.

1.12.2 Modelling of Bacteria and Bacterial Biofilms

Bacterial chemotaxis (e.g. Bray et al. 1993) and bacterial metabolism (reviewed in Covert et al. 2001) have been modelled, but the main focus of bacterial modelling has been on growth; because of its importance in the food industry (to predict microbial safety, Zwietering et al. 1990), in wastewater treatment (Cabrero et al. 1998) and in the production of useful chemicals (e.g. biofuels, Carlozzi et al. 2010). This focus on growth has also extended to bacteria growing as biofilms.

The first biofilm models were developed in the 1970s to explain substrate flux into the biofilm based on substrate utilisation and mass transport (LaMotta 1976, Williamson and McCarty 1976, Rittman and McCarty 1981). During the 1980s the complexity of the models increased to allow multiple microbial types that could be distributed irregularly (Kissel et al. 1984, Wanner and Gujer 1984, Rittman and Manem 1992). Also during the 1980s models were developed to recreate the patterns formed by biofilm growth using diffusion-limited aggregation models (Witten and Sander 1981, Meakin 1983). Then in the 1990s the model started to become even more complicated in attempts to determine the factors controlling the elaborate biofilm morphologies observed using Confocal laser scanning microscopy (Wimpenny and Colasanti 1997; Picioreanu et al. 1998a, 1998b, 2001, 2004; Kreft et al. 1998, 2001; Xavier et al 2005; Alpkvist et al. 2006; Batstone et al. 2006; Wanner et al. 2006; Matsumoto et al. 2007). The recent models can produce complex and detailed descriptions of biofilm geometry and ecology but are computationally intense. Some recent models are so complex that they even model the viscoelastic nature of the biofilm (Klapper et al. 2002) and drag on biofilm streamers (Taherzadeh et al. 2010), however improved versions of some of the early models are still used if they are the most appropriate for the hypotheses under investigation (e.g. logistic models used by Juneja et al. 2009).

The early models were appropriate for the questions they were applied to but as more was asked of the models they increased in complexity. Similar to Einstein's famous quote, *Everything should be made as simple as possible, but not simpler*, a 'golden rule' of modelling is that *a model should be as simple as possible, and only as complex as needed* (Wanner et al. 2006).

1.12.3 Succession of Models

The models used in this study are a logical progression of some of the main models used to describe growth and the interaction of two species.

1.12.3.1 Exponential growth

In the late 18th century, Thomas Malthus developed a model to describe population growth that is now called the Malthusian model of exponential growth (Malthus 1798). The simplicity of the model makes it very useful for predicting population size during the early stages of population growth, although it is of little use during the later stages as the model allows for a constant rate of growth indefinitely, which is not possible due to competition for resources and territory, and the accumulation of waste and increased susceptibility to disease at higher populations.

$$\frac{dP}{dt} = rP$$

$$P(t) = P_0 e^{rt}$$

The only variables are P_0: the initial population, r: the growth rate (which because of the importance of this early model is often called the Malthusian Parameter in this and other more recent models) and t: time.

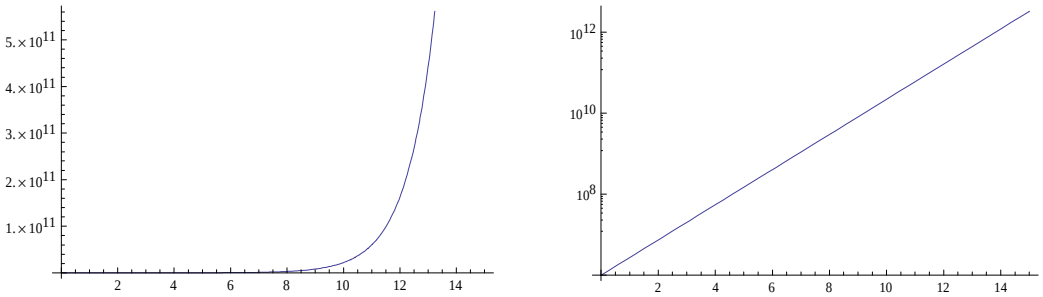


Figure 1.N Plot and Logplot of the exponential growth curve.

Figure 1.N shows the rapid and continuous increase in population size, which is observed in the early stages of population growth, for example after the inoculation of a bacterium into a suitable broth.

1.12.3.2 Logistic growth

This model led to the development of the Logistic Function by Pierre Verhulst in 1838. In this model Verhulst accounted for the limits placed upon the maximum attainable population size that are found in nature.

$$\frac{dP}{dt} = rP(1-\frac{P}{K})$$

The only variables are P: the population, r: the growth rate (Malthusian Parameter), K: the carrying capacity, and t: time. This model is used widely in microbiology, ecology and medicine (for example to model tumour growth, Vaidya and Alexandro 1982). It is very similar in form to the model for exponential growth but it includes K to account for the maximum carrying capacity.

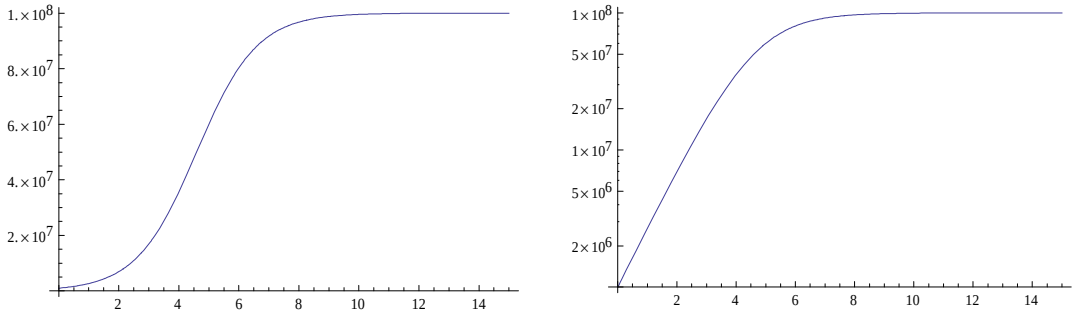


Figure 1.O Plot and Logplot of the logistic growth curve.

Figure 1.O shows the distinctive sigmoidal form which encompasses exponential growth, declining growth and stationary phase. These three phases of growth are contained in the simple equation for logistic growth. In addition to the logistic function, other forms have been developed to describe sigmoidal growth (e.g. Gompertz, Richards, Schnute, and Stannard; Zwietering et al. 1990). These are not described further here as the logistic function is simple to apply and describes the pattern of growth adequately. However it does not describe the death phase.

1.12.3.3 Lotka-Volterra Predator-Prey Equations

The two models described above model the growth of a population but do not describe how a population may decrease in size or how it could interact with another

species. The Lotka-Volterra Predator Prey equations, which were developed independently by Alfred Lotka and Vito Volterra in the 1920s, describe how two species interact, with one species predating on the other, such that both populations go through cycles of growth and death (Takeuchi 1996).

$$\frac{dx}{dt} = x(\alpha - \beta y)$$

$$\frac{dy}{dt} = -y(\gamma - \delta x)$$

In these equations y is the number of a predator with x is the number of its prey and α, β, γ and δ parameters that represent the interactions that occur between the predators and their prey.

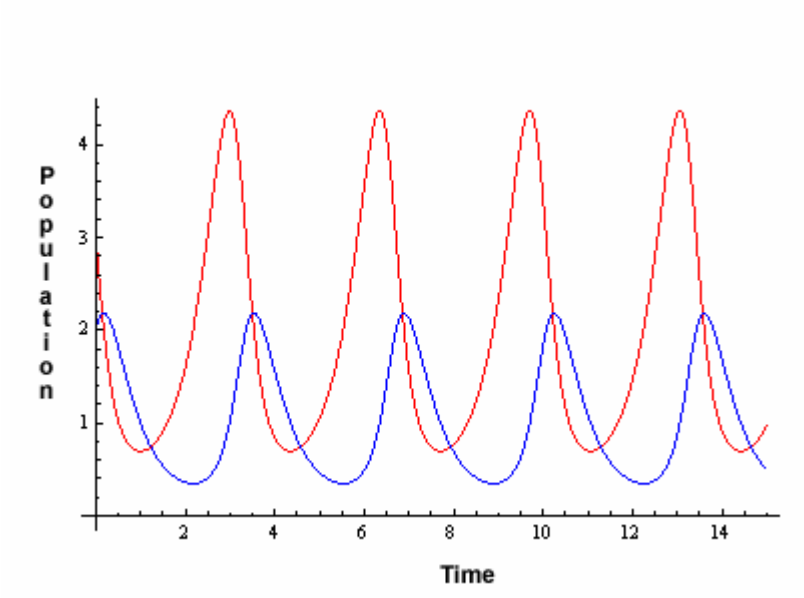


Figure 1.P Plot of Lotka-Volterra Predator-Prey equations.
In this example α=2, β=2, γ=2 and δ=1. The population of the predator is shown in blue and its prey is shown in red.

The coupling of the two species, by factoring in the population size of the other species in the rate of change of each population, produces a cyclical pattern of growth and decline for the two species which can be clearly seen in Figure 1.P.

1.13 Gene Expression

The possibility of investigating interactions at a genetic level makes bacteria ideal study organisms for the investigation of species interactions. Many processes occur in the growth, development and decline of biofilms than can not be studied by quantifying the number and vitality of bacteria, determining the morphology of the biofilm or by determining the pH of the surrounding environment. However, some of these processes can be investigated by studying bacteria at the genetic level using microarrays (Schena et al. 1995). Genes involved in biofilm formation and other social behaviours can be investigated using microarray analysis. Microarray analysis is a high throughput technology for quantifying either genomic DNA or mRNA. Transcribed RNA from the organism under study can be converted into cDNA, bound to a fluorescent marker, washed over an array of synthesised single stranded DNA, and any regions that are complementary will bind. This method can quantify the expression level of every single gene as genes that are expressed will cause the corresponding region of the array to fluoresce in proportion to the original amount of RNA. Microarrays can provide data to indicate how cells are coordinated and they can highlight differences in how cells are coordinated under different conditions. Microarrays are grids of thousands, or sometimes even millions, of short specific oligonucleotide repeats that have been printed on to glass or plastic. The specific sequences can be chosen to perform a variety of comparisons including diagnostic screening for many different diseases, cancer phenotypes and heritable traits, but in this and many other studies they are used to screen gene expression within an entire genome. This is done by identifying what RNA is transcribed within a population of cells. This RNA controls how the cell will proceed, and is an instantaneous sample, a snapshot, of the orders that control the physiology of the cell. Consequently, if bacteria subject to different conditions are profiled using microarrays, the changes effected by these different conditions can be identified. The Pathogen Functional Genomics Resource Center (PFGRC) at the J. Craig Venter Institute (JCVI) provides glass slide microarray resources to successful grant applications. They have kindly awarded microarray slides for this study which display 1948 *S. mutans* ORFs (see Appendix 1 for successful Grant Proposal).

Cells are coordinated by a complex series of chemical reactions. Signals reach the DNA causing specific areas of the DNA to be transcribed in to mRNA. The signals that react with the DNA to initiate transcription are of two types; general transcrip-

tion factors (such as the sigma factor σ^{70}, RpoD) and specific transcription factors (such as ComE) and these work together to mediate the expression of genes by binding to very specific sequences of regulatory DNA called transcription factor binding sites (Venancio and Aravind 2009). This binding does not always act to initiate transcription and in some cases it may act to prevent it as the binding of some specific transcription factors prevents the binding of RNA polymerase. Transcription factors may act at a single site controlling a single gene, a single site controlling multiple genes (an operon) or at multiple sites controlling multiple genes (a regulon). Once the DNA is transcribed into mRNA, it is then translated in to protein. Some of these proteins may themselves act as signals while others function as enzymes controlling reactions that occur within the cell. An example of this is quorum sensing, where gene expression is controlled in response to cell density (Bassler 1999). Another example of a very carefully controlled system, where many genes are activated and repressed is metabolism. Metabolism is a highly regulated and very important network of reactions within the cell. Metabolic pathways are series of enzymatically controlled reactions and can be regulated by controlling the amounts of the different enzymes present. Which enzymes are present, and to what extent, depends upon which metabolic genes are transcribed into RNA. Thus RNA by coding for the enzymes determines how catabolism and anabolism proceed. Catabolic degradation produces energy allowing the cell to function but many of the by-products and intermediates perform useful functions for the cell.

The common evolutionary origins of all organisms and constraints imposed by the laws of thermodynamics on what reactions can occur have resulted in the principles that govern metabolism to be the same for all species. This has resulted in the core of the network of reactions showing strong similarity between very different species and the variations that do occur are due primarily to differences in the nutrients and free energy the species have evolved to exist on. The metabolic pathways for the catabolism of carbohydrates, lipids, and proteins converge on a few common intermediates. This is very useful as enzymes are conserved across species (and Kingdoms) allowing the purpose of an enzyme to be inferred based upon similarity with enzymes in related species. The conservation of enzymes has also led to the conservation of the networks of linked enzymatic reactions. Thus much can be inferred about a species once its genome has been sequenced, as the links of the networks, the enzymes, are known.

The genomes of fourteen species of oral bacteria have been sequenced (www.oralgen.lanl.gov). Because of the clinical importance of *S. mutans*, its genome was the first to be sequenced (Ajdić et al. 2002). The *S. mutans* genome comprises just over 2 million base pairs which encode 1960 genes (Ajdić et al. 2002). In contrast the free living bacterium, *Escherichia coli* has more than twice as many genes (4289 genes and 4.6 million base pairs in the smallest *E. coli* genome sequenced to date, Blattner et al. 1997, www.jcvi.org). As *S. mutans* is an obligate parasite, rather than a free-living bacterium like *E. coli*, it is dependent on its host for the provision of many essential molecules, and thus a large portion of its genome (15%) is devoted to transport systems so that it can acquire, rather than make, essential molecules (Russell 2008). *S. mutans* is a very successful bacterium despite living in a dynamic environment where food abundance, food type, pH and community composition change dramatically. *S. mutans* turns this variability to its competitive advantage and it is through controlling the genes it expresses that *S. mutans* is flexible enough to thrive in these conditions. As there are so many genes in even the simplest of genomes, it is useful to classify and study groups of genes, and thus genes are grouped into role categories based upon similar function. Of the 1960 genes in the *S. mutans* genome; 1492 have been assigned a role category, 93 genes are identified but have not been assigned a role category, 207 are conserved hypothetical genes and 168 are hypothetical genes (www.jcvi.org). Genes and the proteins they code for can be further classified based upon homology into clusters of orthologous groups of proteins (COGs) within which proteins have similar functions (Tatusov et al. 1997) and they correspond to ancient conserved domains (as by definition the proteins need to be present in at least three major phylogenetic lineages).

Ajdić et al. (2002) sequenced the entire genome of *S. mutans* and identified the processes this species employs by comparing its genome sequence against databases of known sequences. They looked for homology at the genetic level to infer the function of the genes *S. mutans* possesses. Having the entire genome sequenced facilitated the development of microarrays which in turn facilitate the analysis of the extent of expression of these genes under different conditions, helping to further elucidate the role of different genes and determine the way they work together to allow *S. mutans* to thrive and survive. Studies have been done on gene expression in *S. mutans* that do not use microarrays (e.g. investigating competence development

and stress tolerance using RT-PCR, Ahn et al. 2006) but microarrays are particularly useful as they can investigate the entire genome simultaneously.

S. mutans microarrays and mutant strains of *S. mutans* have been used to investigate bacteriocin production and regulatory pathways (Merritt et al. 2005b), biofilm formation (Wen et al. 2006), signal recognition and physiological adaptation (Hasona et al. 2007), carbohydrate transport and use and virulence gene expression (Abranches et al. 2006, Abranches et al. 2008), the stringent response and the nutritional alarmones (Lemos et al. 2008), and stress sensing (Zhang and Biswas 2009). Using wild-type *S. mutans*, microarrays have been used to investigate the role of HtrA (a surface protease, Biswas and Biswas 2005), morphology and biofilm formation of clinical strains (Motegi et al. 2006), the effect of manganese on virulence (Arirachakaran et al. 2007), carbohydrate transport and use (Ajdić and Pham 2007), the quorum sensing role of LuxS in contrast to its role as part of the methyl cycle (Sztajer et al. 2008), the stringent response and global control of gene expression (Nascimento et al. 2008), exposure to oxygen (Ahn et al. 2007), signalling and stress (Biswas et al. 2008), acid-inducible genes (Gong et al. 2009), differences in expression between planktonic and biofilm phenotypes (Shemesh et al. 2007), differences in expression at different thicknesses of biofilm (Shemesh et al. 2008) and between single and dual-species biofilms (Luppens et al. 2008). This last study is similar to this one, but using a different species of veillonellae, *V. parvula*.

Of particular interest are genes involved in biofilm formation, acid tolerance response, sugar transport and metabolism, and communication within and between species. Because this study investigated two species that are coupled around lactic acid, the glycolysis pathway was of interest as lactic acid is one of the final metabolic products of this pathway. Also signalling networks, extracellular structures and defence mechanisms are of key interest as they are other obvious candidates of ways the two species may interact.

Typically studies investigating interactions sample a single point in time and do not investigate if the dynamics of the interactions change over time. This study sets out to investigate from ecological to genetic levels how *S. mutans* and *V. dispar* interact in planktonic culture and as biofilms over a period of time covering the establishment, maintenance and decline of the systems.

1.14 Metabolic Network of the Plaque Biofilm

The nutritional and environmental conditions required for bacterial growth are well understood for some species but there are many species that are unculturable in a laboratory environment, indicating how specific can be the environmental niches that exist *in vivo*. Many unculturable bacteria may need to associate with other bacteria for their particular niche to exist. A bacterial species may need the waste product of a different species for energy but it may also need its own waste products removed from the system, before they accumulate to toxic levels, by yet another bacterial species. There may be many sets of interactions like this occurring concurrently with each species taking different roles in different sets (see Figure 1.Q).

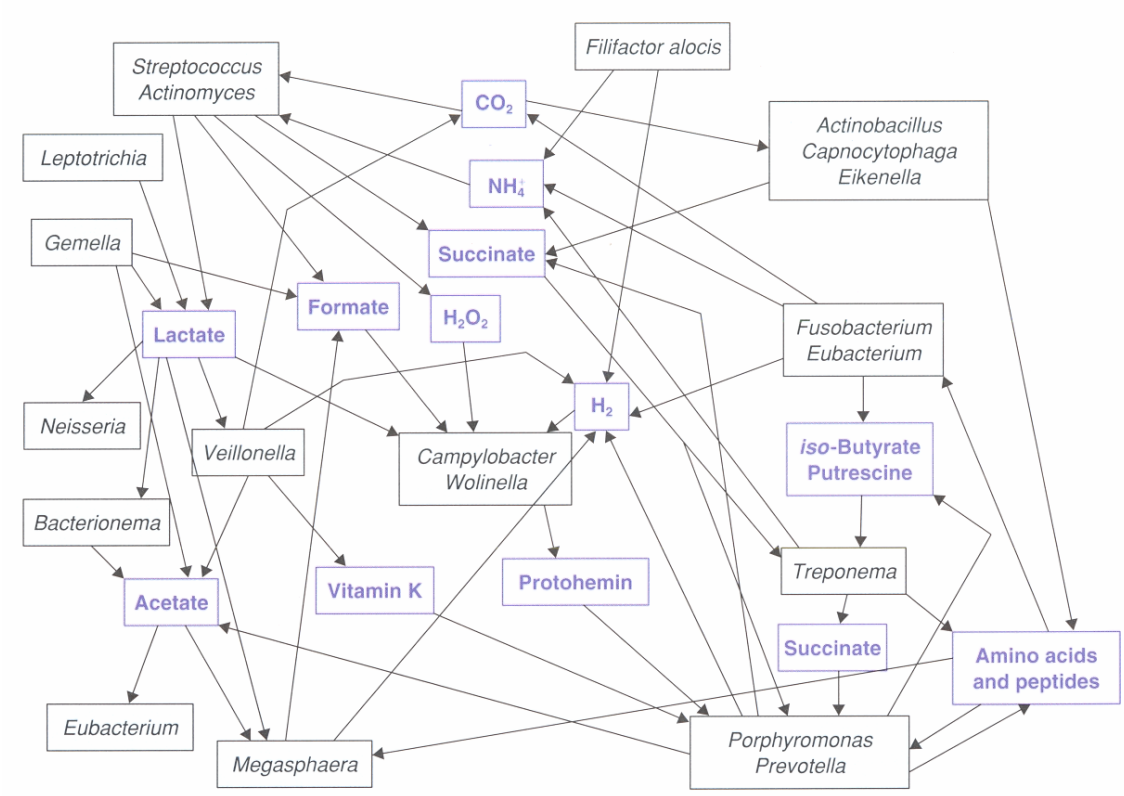


Figure 1.Q Nutritional interactions that occur between organisms in the oral cavity. These interactions are putative but serve to show a possible subset of the complex food-web that is present in the oral cavity. One organisms waste products are another organisms nutrients and these nutrients are shown in blue in this figure. This study focuses on a small subset, *Streptococcus*-Lactate-*Veillonella*, of this complex network. Reproduced with permission from [John Wiley and Sons Ltd] (Wilson 2008), copyright (2008).

There are many metabolic pathways utilised by bacteria and consequently different bacterial species require many different combinations of nutrients. Some bacteria can synthesise all of their components when provided with sources of energy and certain elements (primarily C, O, N, H, P, S, but also K, Mg, Ca, Fe, Zn and Mn) while other bacteria need many of their amino acids provided ready-made as they have dispensed with biosynthetic pathways that are redundant in the niche they live in (Prescott et al. 1996). *In vivo,* bacteria compete with each other for nutrients and produce waste products that are nutrients for, or toxic to, other bacteria. There are many highly specific stratified environments in the oral cavity providing all sorts of nutrients and environmental niches that are created by the complex bacterial communities living in the mouth (see Figure 1.R). As a consequence of the abundant carbohydrate supply provided by food impacted in fissures, *S. mutans* are a dominant member of the biofilm community found at this site (see Figure 1.R.a), which directly leads to fissures being the most common site of caries (Wilson 2008). The climax communities on approximal surfaces (between the teeth) and on smooth surfaces differ from the climax community found in fissures, and from each other, due to their differing environmental determinants, although streptococci and *Actinomyces* spp. are the predominant cultivable bacteria found at all three sites and *Veillonella* spp., while not the predominant genus, are found at all three sites (Bowden et al. 1975, Theilade et al. 1982, Beighton et al. 1999, Wilson 2008). It is estimated that the vast majority, 98%, of all bacteria can not be cultured in the laboratory (Wade 2002). This estimate of unculturable bacteria will be increased further following the study by Keijser et al. (2008) that sequenced ribosomal subunits and found vast numbers of previously unidentified phylotypes in the oral cavity, but it serves to show, that the nutritional requirements of identified bacteria are very hard to recreate. Some of the unculturable bacteria will be unculturable because the right conditions have not yet been determined, some will be unculturable without other species present and some will be unculturable because their environmental and nutritional requirements are not yet known.

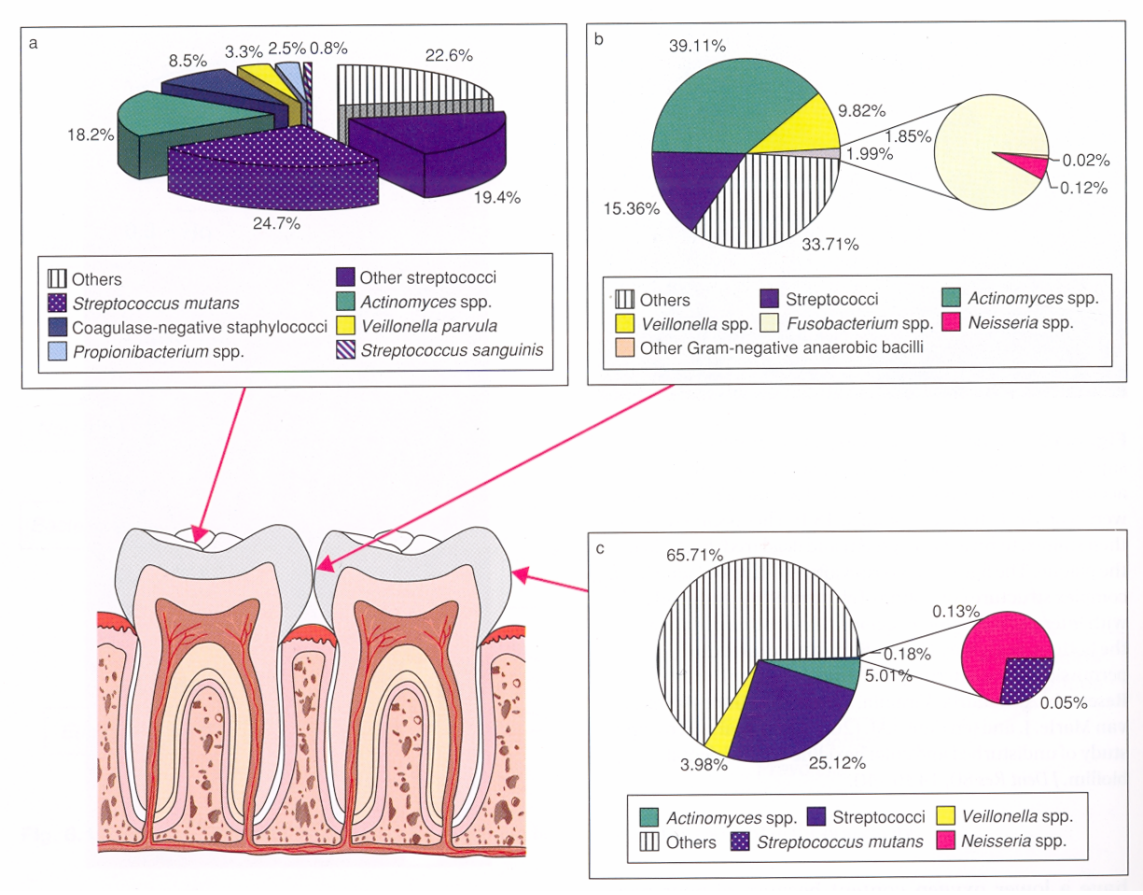


Figure 1.R Diagram showing the predominant cultivable microbiota, and where they are found, of the three main types of supragingival plaque: a-live in fissures, b-approximal (live between the teeth) and c-smooth surface. Data combined from three studies sampling a total of 40 healthy adults (Bowden et al. 1975, Theilade et al. 1982, Beighton et al. 1999). Reproduced with permission from [John Wiley and Sons Ltd] (Wilson 2008), copyright (2008).

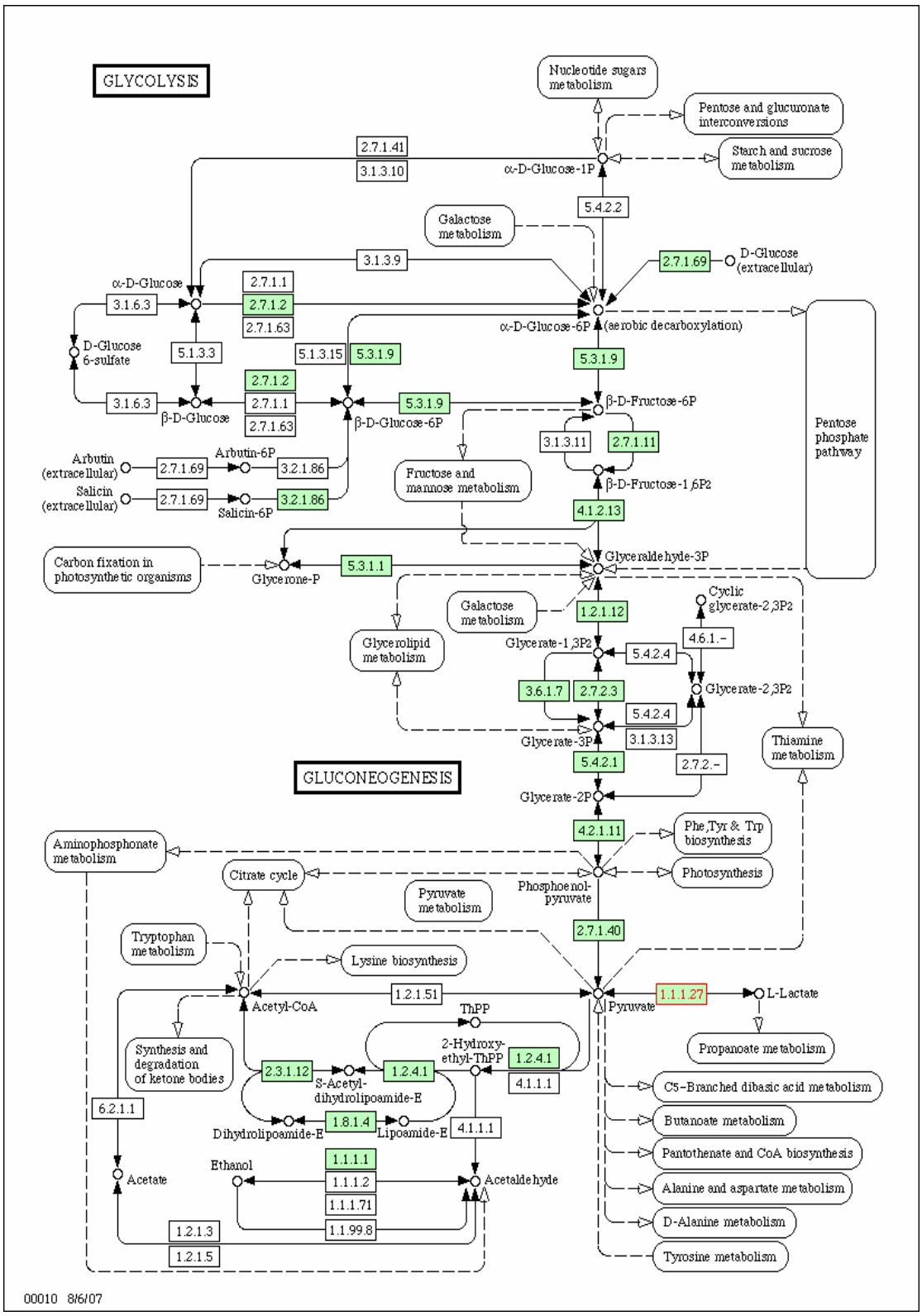


Figure 1.S KEGG glycolysis/gluconeogenesis reference pathway showing the molecules and enzymes used in the breakdown of sugars including lactate dehydrogenase. Lactate dehydrogenase is the final enzyme in the pathway that converts sugars into lactic acid. Image reproduced from Kyoto Encyclopaedia of Genes and Genomes. http://www.genome.jp/dbget-bin/show_pathway?smu00010+SMU.1115

Analysis of the genes being expressed by *S. mutans* under different experimental conditions and stages of growth will identify genes involved in biofilm formation and other social behaviours. As cooperation between *S. mutans* and *V. dispar* is predicted based upon them having a shared metabolic pathway, any changes in gene expression between single and dual species biofilms of genes involved in the *S. mutans* glycolysis/gluconeogenesis pathway are of particular interest (see Figure 1.S).

In this study biofilms were prepared by inoculating filters on agar with both or either of *S. mutans* and *V. dispar* and enumerating the relative numbers of each organism growing. The global transcriptome of *S. mutans* was assayed to identify what differences, if any, resulted from growing as a dual species biofilm with *V. dispar*. Simple mathematical models were developed and compared against the findings of the experimental studies to make testable predictions. This was done to uncover the roles of cooperative versus competitive interactions, and other potential strategies such as altruism and cheating in the successional formation of biofilms. The literature states that these two species display a mutualistic relationship based around the production and removal of lactic acid so this thesis reports on studies trying to determine whether these bacteria have developed methods to facilitate this interaction and whether the types of interactions that occur could suggest novel non-antibiotic means of promoting bacterial mutualisms and controlling bacterial disease.

1.15 Aims

(i) to characterise the formation, growth, maintenance and decline of planktonic cultures and biofilms comprising *S. mutans* and *V. dispar* in isolation and combination.

(ii) to investigate the effects these two species have on each other when grown as dual-species cultures and biofilms.

(iii) to investigate the effects the cultures and biofilms have on the pH of the surrounding environment and the effect buffering the pH of the environment to be acidic, neutral and alkaline has on the growth of, and interactions between, the species.

(iv) to investigate how competitive and cooperative interactions in a mixed species biofilm alter the global transcriptome of *S. mutans*.

(v) to integrate the data collected into models of the interactions of these two species growing as biofilms.

Hypothesis: *S. mutans* and *V. dispar* will benefit from the presence of the other species because the waste product of *S. mutans* is a nutritional source for *V. dispar*.

2 Materials and Methods

2.1 Bacterial Species

Streptococcus mutans UA159 and *Veillonella dispar* (clinical isolate) were sampled from stocks provided by Dr Derren Ready (UCL Eastman Dental Institute). These samples were streaked on Tryptone Soya Agar plates (Autoclaved at 115ºC for 15 minutes to prevent the D-glucose caramelising, Tryptone Soya Broth [Soybean-Casein digest medium U.S.P., CMO129] and 1.5% Agar Bacteriological medium [Agar No. 1, LP0011], Oxoid, Basingstoke, England), grown for two days under anaerobic conditions (10% carbon dioxide, 10% hydrogen, 80% nitrogen, MACS-MG-1000 anaerobic workstation, Don Whitley Scientific Ltd., Shipley, England) and verified by colony morphology and microscopically using the Gram-stain as being the correct morphotype. Glycerol stocks were made for both species for all future experiments. Using a sterile culture loop, a single colony was sampled and streaked on TSA plates as a lawn (contamination is easily visible and large numbers of bacteria are grown) and grown as before and this was done twice for each species. After two days of growth, 1ml of Tryptone Soya Broth (Soybean-Casein digest medium U.S.P., CMO129, Oxoid, Basingstoke, England) was pipetted onto the lawn and using the side of a sterile culture loop all the bacteria were scraped off the agar to go into suspension in the TSB. This was then pipetted up and put into a labelled 2ml screw-cap tube containing 0.5ml of glycerol (added earlier using a wide tip pipette). This was then mixed using a wide tip pipette and stored at -80ºC.

2.2 Bacterial Growth

Bacteria were grown planktonically (in liquid culture medium) and as biofilms (on nitrocellulose filters on agar).

2.2.1 Planktonic Growth

Two experiments were conducted investigating the interaction between *S. mutans* and *V. dispar* in unbuffered planktonic culture and in buffered planktonic culture (approximately neutral [7.2], acidic [5.1 and 6.3], and alkaline pH [8.1]). Medium was buffered using 0.2M acetate buffer (0.1M acetic acid [Fisher Scientific, Loughborough, England] and 0.1M sodium acetate [BDH Laboratory Supplies,

Poole, England]), 0.4M MES buffer, 0.4M MOPS or 0.4M Tricine buffer (Sigma, www.sigmaaldrich.com) and titrated to adjust to the desired pH using KOH pellets (BDH Laboratory Supplies, Poole, England). *S. mutans* and *V. dispar* were grown from glycerol stocks and verified before being grown in 50ml TSB for 17 and 72 hours respectively under anaerobic conditions. Unbuffered and buffered TSB was inoculated with 10^6 colony-forming units (cfu) of either or 10^6 cfu of each of both species and the development of the cultures was monitored by measuring optical density, total viable counts, live/dead ratios and pH over a fourteen day period. The unbuffered and buffered experiments were carried out in triplicate and conducted twice (although the acetate buffering was only conducted once as it was included as a response to the data collected in the first cohort).

2.2.1.1 Planktonic growth - Optical Density

Optical density readings were taken at wavelengths of 590nm and 650nm, of 200µl samples removed from the planktonic cultures, using a Dynex MRX*TC*ii plate reader (Prior Laboratory Supplies, East Sussex). Optical density was measured at two wavelengths to investigate the possibility of developing simultaneous equations to calculate the relative density of each species in dual species cultures but the absorbance of each species at these wavelengths did not differ sufficiently, thus only readings of 590nm are referred to throughout the rest of this thesis. All samples were scanned after one, three, seven and fourteen days and samples of one of the three replicates, rotating each day, were taken every day and at 0.25 days. Sampling just one of the three replicates every day reduced the possibility of contaminating the cultures, while ensuring data was collected throughout the entire time-course.

2.2.1.2 Planktonic growth - total viable counts of bacteria

The planktonic cultures were serially diluted and plated out on tryptone-soya plates. Vancomycin (6µg/ml) was added to half of the plates for the selective growth of *V. dispar* which were then grown anaerobically and the other half of the plates (containing no antibiotic) were grown in carbon dioxide (5% in air, MkII Proportional Temperature Controller, Leec, Cardiff, Wales) for the selective growth of *S. mutans*. Colonies were counted using a plate counter (Anderman Colony Counter, Anderman, Kingston-Upon-Thames, England). *V. dispar* were grown on plates with and without

vancomycin in order to ascertain whether this made a difference to the number of bacteria recovered and it did not (two tailed paired t-test, t=0.611, d.f.=7, p=0.561). *S. mutans* was grown in carbon dioxide and anaerobically to ascertain whether this made a difference to the number of bacteria recovered and it did not (two tailed paired t-test, t=0.566, d.f.=7, p=0.589). The colonies of the two species are easily identifiable and no *S. mutans* colony was ever observed growing on a plate containing Vancomycin or *V. dispar* colony observed growing on a plate incubated in carbon dioxide.

2.2.1.3 Planktonic growth – pH

pH readings of 500µl samples removed from the planktonic cultures were taken using an Orion Tris Calomel pH Electrode (7102BN, Thermo Fisher Scientific Ltd., Loughborough, England). All samples were measured at one, three, seven and fourteen days and samples of one of the three replicates were taken every day and at 0.25 days.

2.2.1.4 Planktonic growth – vitality

Bacterial vitality was determined using fluorescence microscopy and fluorescent stains that indicate whether the bacteria are functioning. 0.5µl of both LIVE/DEAD *Bac*light Bacterial Viability Kit component A and component B (Catalogue number L7007, Invitrogen Ltd, Paisley, U.K.) were added to 180µl of a mix of broth diluted 1 in 10 with saline and this was left for 15 minutes in the dark to allow the staining to occur. 20µl of this mix was put on a slide, and a coverslip was placed on top. The bacteria were visualised using an Olympus BX51 microscope with UPlanFLN 100x oil immersion objective, U-LH100HG Mercury Burner, QImaging MicroPublisher 5.0 RTV camera and SimplePCI (Version 6.0.0.060805; Compix Inc.) software. The numbers of live (green) versus dead (red) bacteria were counted. Because of the enormous processing time required to conduct the buffered experiment, vitality was only able to be investigated in the unbuffered experiments.

2.2.2 Single and Dual Species Biofilms

This study uses a multi-pronged approach to try to unravel the processes occurring in the formation and maintenance of the dental plaque biofilm. Biofilms of *S. mutans* by

itself, *V. dispar* by itself and the two species together were grown using the 'membrane reactor' method (Wimpenny 2000). The biofilms were grown under controlled conditions and data on the numbers of bacteria after set time intervals, the proportion of live versus dead bacteria in the biofilms and the pH of the biofilms were collected. The biofilms were also visualised and their structures examined. Data on the genes being expressed by *S. mutans* in the different biofilms was collected. Three different biofilm growth experiments were conducted; an investigation into the growth of *V. dispar* and *S. mutans* biofilm when *S. mutans* outnumbers *V. dispar* 10 to 1 (10 *S. mutans* to 1 *V. dispar* experiment, similar to what would occur in dental plaque when *V. dispar* is a secondary coloniser into an already established *S. mutans* biofilm), a continuation of this experiment with an investigation in to the effect of relative inoculum size on the interaction between *S. mutans* and *V. dispar* in biofilms (1/10 to 1, 1 to 1, 1 to 1/10, biofilm inoculum size experiment) and an investigation into the effect of D-glucose concentration on the interaction between *S. mutans* and *V. dispar* in biofilms (biofilm D-glucose concentration experiment).

2.2.2.1 Single and dual species biofilms - biofilm growth model

Preliminary experiments were conducted to determine methods of growth and sampling as outlined in Section 3.2 but the methods used for the main experiments are outlined in detail here. 10ml of TSBA (Autoclaved at 115ºC for 15 minutes to prevent the D-glucose caramelising, 0.3g TSB medium and 0.15g Agar Bacteriological medium, Oxoid, Basingstoke, England) was pipetted into each well of six-well tissue-culture plates (flat bottom with lid, catalogue number 83.1839, Sarstedt, Newton, U.S.A.). In the D-glucose concentration experiment the agar was made using the constituent ingredients of TSA but without D-glucose ([17g/L Tryptone X LP0642, 3 g/L Neutralised Soya Peptone LP0044, 15g/L Agar No.1 LP0011, Oxoid, Basingstoke, England], [5g/L sodium chloride, 2.5g/L dibasic potassium phosphate, BDH Laboratory Supplies, Poole, England]) and D-glucose (dextrose, BDH Laboratory Supplies, Poole, England) was filter sterilised and added subsequent to autoclaving to make agar with the desired D-glucose concentrations (0, 0.025, 0.25, and 2.5 g/L). Additionally, TSBA was made with 25g/L D-glucose added prior to autoclaving because adding that amount of filtered D-glucose after autoclaving would significantly lower the concentration of the other ingredients in the agar. Nitrocellulose filters (Cellulose Nitrate Membrane Filters, 0.2µm pore size, 13mm

diameter, Catalogue number 7182-001, Whatman International Ltd., Maidstone, England) were placed onto the middle of the agar using sterile tweezers (see Figure 3.A). Each filter was on agar 30mm in diameter and 5mm thick (see Figure 3.A).

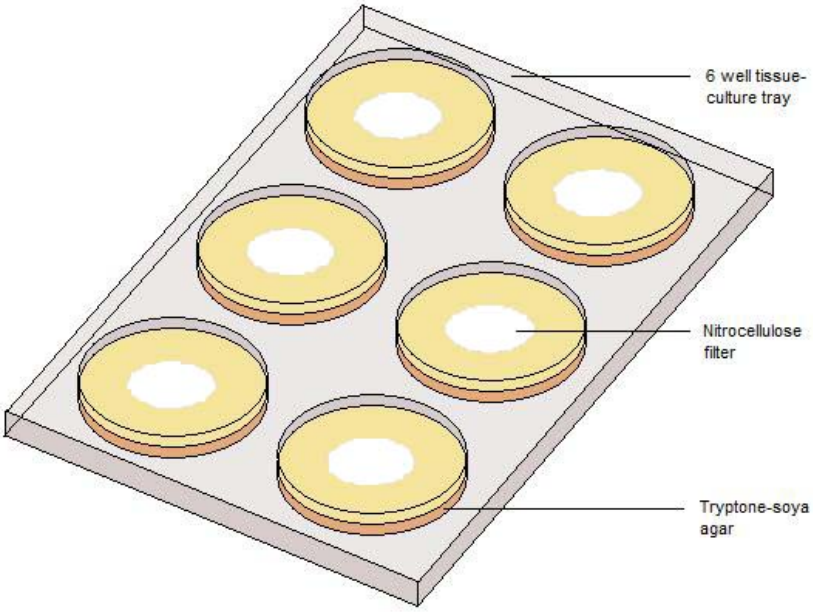


Figure 2.A The experimental setup for growing six biofilms simultaneously.

S. mutans and *V. dispar* were grown from glycerol stocks and verified before being grown in 50ml TSB for 17 and 72 hours respectively under anaerobic conditions. Cultures were adjusted to the desired concentration of bacteria based upon OD590 readings taken with a spectrophotometer. 10µl of either or both cultures was pipetted on to the middle of the nitrocellulose filters to seed the biofilms.

The inoculate was allowed to settle for one hour before the tissue culture trays were inverted and the biofilms were left to grow under anaerobic conditions for one, three, seven and fourteen days and investigated as outlined in Figures 2.B and 2.C. Negative controls were used for all replicates of this procedure.

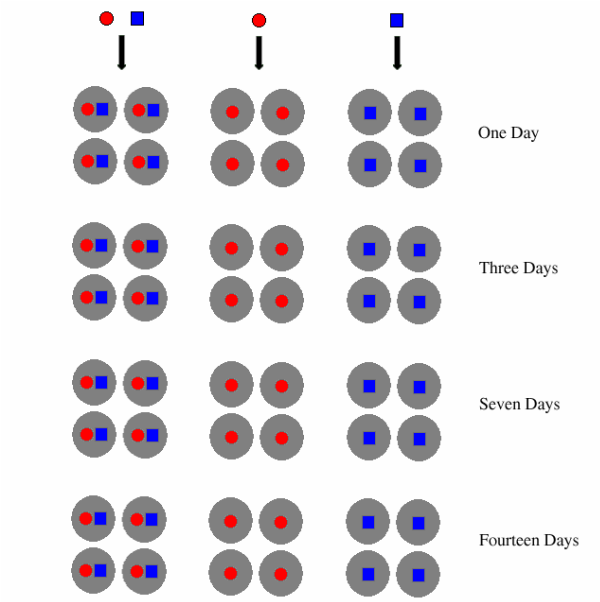
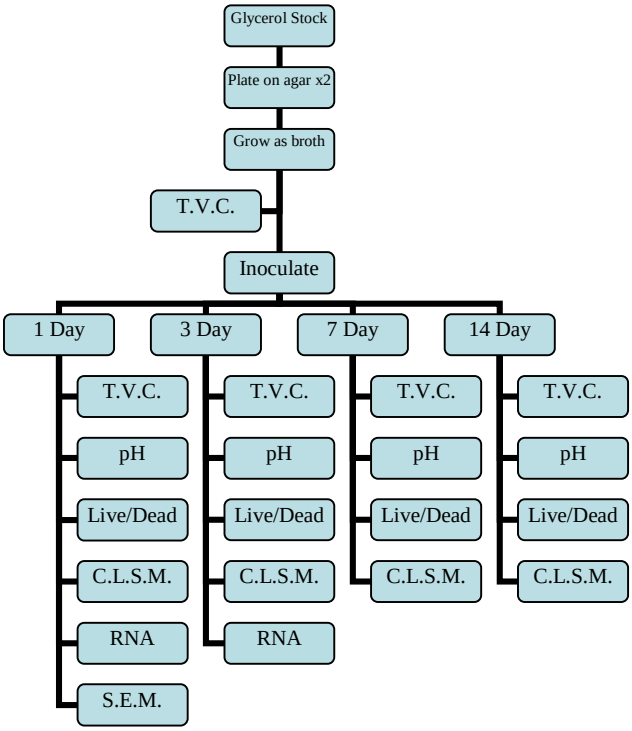


Figure 2.B Schematic showing one cohort of a biofilm growth experiment. The grey circles represent nitrocellulose filters and the red circles and blue squares represent the different species. A red circle indicates *S. mutans* was inoculated on to the filter. A blue square indicates *V. dispar* was inoculated on to the filter. This shows the three types of biofilms grown for each time point that were assayed for total viable counts, pH and vitality. In addition to these, filters inoculated with sterile broth were incubated for each time point as negative controls and one of each biofilm type for each time point was inoculated for Confocal analysis. This details the setup for one cohort (there were four) of the 10 *S. mutans* to 1 *V. dispar* biofilm experiment.

2.2.2.2 Single and dual species biofilms - growth and screening protocol

Biofilms were sampled at one, three, seven and fourteen days with measurements taken of total viable counts, pH, and vitality. Biofilm morphology was also investigated as outlined in Section 4 and gene expression was investigated as outlined in Section 5 (see Figure 2.C).



T.V.C.	total viable counts of bacteria
pH	measurements of pH
Live/Dead	Fluorescence microscopy of bacteria using Live/Dead stain (vitality)
C.L.S.M.	Confocal laser scanning microscopy of biofilms (structure and vitality)
S.E.M.	scanning electron microscopy of biofilms (morphology)
RNA	storage and analysis of messenger RNA (gene expression)

Figure 2.C The experimental protocol employed in the analysis of the interactions of *S. mutans* and *V. dispar* in bacterial biofilms.

This figure provides an overview of the experimental protocol employed in the analysis of the bacterial biofilms. It shows the preperation and analyses done after each length (one, three, seven and fourteen days) of biofilm growth.

2.2.2.3 Removing bacteria from nitrocellulose filters and disrupting aggregations of bacteria

Nitrocellulose filters with the biofilms growing on them were removed from the wells and placed in labelled 25ml tubes containing 10ml of normal (0.85%) saline. These were then vortexed for two minutes (maximum speed, WhirliMixer, Fisher Scientific, www.fisher.co.uk).

2.2.2.4 Single and dual species biofilms - total viable counts of bacteria

Once bacteria were removed from filters, total viable counts and selective growth of each organism were conducted as described in the earlier section on total viable counts of planktonic cultures.

2.2.2.5 Single and dual species biofilms – pH measurements

Once the filters had been removed, triplicate readings of the pH of the agar that was immediately under the filter were made using a flat-surface pH meter (Orion

8135BN Thermo ROSS flat surface pH meter, Thermo Fisher Scientific Ltd., Loughborough, England). Readings of pH of biofilms growing on filters were taken and compared against readings of the agar underlying the filter to ascertain if taking readings of the agar accurately measured the pH of the biofilm itself and it did (two-tailed paired t-test, t=0.957, d.f.=4, P=0.393).

2.2.2.6 Single and dual species biofilms – vitality measurements

Bacterial vitality was determined using fluorescence microscopy and fluorescent stains that indicate whether the bacteria are functioning as explained for the planktonic experiments but differing for the biofilm experiments in that the stains were added to 180µl of the original saline containing the bacteria. Because of the intensive processing time, vitality measurements were only taken for the 10 *S. mutans* to 1 *V. dispar* experiment.

2.2.3 Effect of Lactic Acid on Growth of *V. dispar*

The effect of lactic acid on the growth rate and yield of *V. dispar* was tested using a range of lactic acid concentrations, in buffered and unbuffered media (Tryptone soya broth; 0mMol, 25mMol, 50mMol unbuffered and 0mMol, 25mMol, 50mMol, 100mMol, 111mMol, 140mMol, 200mMol lactic acid [lactic acid solution, SR0021K, Oxoid, Basingstoke, Hants, England] buffered using PIPES [50mMol]; concentrations chosen to cover a range and to use concentrations published in the literature; Rogosa 1964, Egland et al. 2004, Kara et al. 2006, Palmer et al. 2006). The experiment was conducted with 200µl of medium in a 96 well plate (flat bottom with lid, Catalogue number 83.1835, Sarstedt, Newton, U.S.A.) with absorbance readings taken at 590nm using a plate reader (MRX TC II, Dynex Technologies Ltd., West Sussex, England). Readings were taken over 94 hours and six replicates were taken for each data point.

2.3 Statistical Analyses

Statistical analyses were carried out using SPSS (Student Version 15.0) and Microsoft Excel (2003). Parametric tests were carried out on all data following numerical and graphical examination of skewness and kurtosis (as on data sets of this size

normality tests provide little useful information). Unless stated otherwise graphs plot arithmetic means with error bars of one standard deviation. Where error bars are plotted on graphs with logarithmic scales, if the lower error bar has a value less than zero, only the upper error bar is shown (logarithmic scales can not show zero or negative numbers accurately). Parametric tests were used to compare means. When doing ANOVA tests, all post hoc comparisons of means are made using Dunnett's T3 method as it does not assume equal variances. Because of the difference in variation of total viable counts between days, all non-zero counts were Log_2 transformed when used in the general linear models unless otherwise stated. Type III sum of squares were used for general linear models as they deal well with unbalanced models with no empty categories.

2.4 Biofilm Structure Studies

Biofilm structure was investigated using Confocal Microscopy and Scanning Electron Microscopy.

2.4.1 Confocal Microscopy

The structure and vitality of biofilms was determined using confocal laser scanning microscopy of biofilms stained with LIVE/DEAD fluorescent stain. 5ml of normal saline (0.85% NaCl) containing 1.25µl each of LIVE/DEAD *Bac*light Bacterial Viability Kit component A and component B was gently poured into each well submerging the biofilms. The tissue culture plate was then wrapped in tin foil for 15 minutes so the dyes could penetrate the biofilm and stain the bacteria without being exposed to light. The staining mix was prepared in advance in a falcon tube (and wrapped in tin foil) so that exactly 5ml of the staining mix could be poured on to the biofilms at the appropriate time as the amount of each dye and the duration of biofilm immersion in the dye would change the fluorescence. Scans were taken of the biofilms using an Olympus BX-51 upright microscope equipped with a Bio-Rad Radiance 2100 confocal laser scanning head, using a 40X water objective (LUM-PlanFI 40Xw). The system was set up to use laser emissions of 488nm (argon) and 543nm (helium neon) to excite the LIVE and DEAD stains respectively. Laser power was set to 5% of total available for both of the wavelengths used, and both pho-

tomultiplier tubes were set to the same levels (PMT1, PMT2: Iris=5.0, Gain=5.0, and Offset=-20.0). Two scans of each field (Kalman=2) were taken and averaged to remove unwanted noise and the step size for the z-series was set to 1 or 2µm (depending upon thickness of the sample and time available). Data files produced were analysed using ImageJ 1.36b (http://rsb.info.nih.gov/ij/). The red image was converted to blue to avoid problems for people who are red/green colour blind. The distribution of cell vitality was determined by constructing fluorescent profiles, which plotted total image brightness for the live and the dead channels against the depth in the image stack (Valappil et al. 2007).

2.4.2 Scanning Electron Microscopy

The morphology of the biofilms was determined using scanning electron microscopy. Nitrocellulose filters with attached biofilm were fixed in 3% glutaraldehyde (Agar Scientific, Essex, UK) in 0.1M sodium cacodylate buffer (Sigma) at 4ºC overnight. The specimens were then post-fixed in 1% osmium tetroxide (Sigma) at 4ºC for two hours and then dehydrated in a series of alcohol concentrations (ethanol, 20%, 50%, 70% and 90%, 15 minutes per concentration). The specimens were then rinsed three times in 100% acetone (10 minutes per rinse), immersed in hexadimethyldisilazane (Taab Laboratory Equipment Ltd., Reading, UK) for two minutes and left to dry in a dessicator. The specimens were mounted on to aluminium stubs (Agar Scientific) using araldite (Sigma) and sputter-coated with gold/palladium in a Polaron E5000 sputter coater. The specimens were viewed using a Cambridge 90B Stereoscan electron microscope (Cambridge Scientific Instruments Ltd, Ely, U.K.) at 15KV.

2.5 Gene Expression Analysis

The genes that were expressed by *S. mutans* growing as a biofilm were investigated, as was the effect of the presence of *V. dispar* on the global transcriptome. 17 microarray slides were hybridised for this analysis (in addition to many in the development of the technique) comprising four sets (*S. mutans* single species biofilms grown for 1 and 3 days and dual species *S. mutans* and *V. dispar* biofilms

grown for 1 and 3 days) of 4 biological replicates and 1 negative control (*V. dispar* DNA and cDNA) using a common reference design methodology.

2.5.1 Grow Biofilms

Single species *S. mutans* biofilms and dual species *S. mutans* and *V. dispar* biofilms were grown for 1 and 3 days as outlined for the biofilm invasion experiment (see Section 2.2.2).

2.5.2 Extraction and Purification of RNA

A range of methods were trialled to extract RNA as outlined in Section 5.2, but the method eventually used is outlined here as it optimised quality and yield while enriching for *S. mutans* RNA when grown as a dual species biofilm.

2.5.2.1 Working with RNA

Ribonucleases (RNases) are enzymes that destroy RNA. They are very stable. They are in the water supply, are produced by microorganisms that live in the air and are produced by humans. RNases can destroy RNA even when they are only present in trace amounts. Thus to work with RNA it is important to do so in an RNase-free environment using RNase-free consumables and reagents.

In addition to using aseptic technique, all RNA work was done in a hood (workspace enclosed on five sides that typically uses airflow to protect the user, or user and experiment from dangerous substances, organism and contamination) that had been cleaned with RNase*Zap* solution (Ambion) to remove RNases, with all consumables and reagents being thoroughly wiped with RNase*Zap* solution before being put in the hood (the hood was switched off to reduce airflow and used instead as a controlled space where all surfaces and consumables could be treated to remove RNAses), or in a dedicated RNA laboratory that was regularly cleaned. All consumables and reagents used were certified RNase free.

2.5.2.2 Protecting and enriching-for *S. mutans* RNA

After one and three days instantaneous samples were taken of *S. mutans* gene expression for *S. mutans* growing as a single species biofilm and as a dual species biofilm in the presence of *V. dispar*. For each sample six filters with biofilms growing on them were placed in a 15ml Falcon tube containing 10ml of 4M GTC solution (GTC Solution: 4M guanidium thiocyanate, 0.4% sodium N-lauroyl sarcosine, 20mM sodium citrate, and 0.8% Tween-80, [made overnight in a warm room as the reaction is endothermic –but not microwaved as this would release cyanide gases] with 0.08M β-mercaptoethanol added just prior to use). This solution stops transcription while preventing RNA degradation. The falcon tubes, containing GTC solution and the six filters, were left for five minutes at room temperature and then vortexed for two minutes to dislodge the bacteria from the filters. The filters were removed using sterile tweezers and the falcons were left for a further 10 minutes at room temperature. The falcon tubes were then spun at room temperature for 30 minutes at 3000g (Eppendorf Centrifuge 5804R) to pellet the bacterial cells and then the supernatant was poured off. GTC solution lyses Gram-negative but not Gram-positive bacterial cell walls which in this study served to enrich for *S. mutans* RNA in the dual species biofilms as once the Gram-negative *V. dispar* bacteria lysed they would not pellet and their RNA was poured off with the supernatant.

2.5.2.3 Cell lysis and RNA purification

The next stage lysed the cell wall and purified the *S. mutans* RNA. For each sample, 1ml of RNAprotect solution (MP Biomedicals) was added to the pelleted cells and they were homogenised by pipetting the solution up and down with the pipette. This solution was then transferred to a Lysing Matrix tube (MP Biomedicals) and processed on a Ribolyser (Hybaid Ltd, Thermo Quest Scientific, Equipment Group, Basingstoke, U.K.) for 40 seconds at a setting of 6.0. The tube was then removed and placed on ice for two minutes before being reprocessed on the Ribolyser for a further 40 seconds (setting 6.0). The tube was then centrifuged at 12000g for five minutes at 4ºC (Eppendorf Centrifuge 5417R). The upper phase was then transferred to a new microcentrifuge tube, taking care not to transfer any cell debris or lysing matrix. This was then incubated at room temperature for five minutes. 300µl of chloroform (Sigma) was added to the tube and this was vortexed for 10 seconds. The tube was then incubated at room temperature for five minutes before being centrifuged at

12000g for five minutes at 4ºC. The upper phase was then transferred to a new microcentrifige tube. 500µl of 100% ethanol (VWR BDH) was added to the tube before gently inverting it five times and placing it at -20ºC for two hours. The tube was then centrifuged at 12000g for 15 minutes at 4ºC and the supernatant was discarded. The RNA pellet was gently washed twice with 75% ethanol (made with nuclease-free water [Qiagen]). The ethanol was then removed and the RNA pellet air dried for five minutes at room temperature. The pellet was then resuspended in 50µl of nuclease-free water. This was incubated for five minutes at room temperature. RNA concentration was determined (see Section 2.5.4) and the RNA was stored at -80ºC.

2.5.3 DNA Extraction

This was done to provide a common reference for the competitive hybridisation in the case of *S. mutans* DNA and to provide a negative control in the case of *V. dispar* DNA. *S. mutans* and *V. dispar* were grown from glycerol stocks and verified before being grown in duplicate in 50ml TSB for 17 and 72 hours respectively under anaerobic conditions. For each sample the tube was centrifuged for 10 minutes at 5000g to pellet the bacteria. The supernatant was discarded and the pellet was resuspended in 0.5ml 120mM Phosphate buffer (pH 8, [94% 1M K_2HPO_4 and 6% 1M KH_2PO_4] + 5% CTAB). This was added to a Lysing Matrix tube and 0.5ml phenol : chloroform : isoamyl alcohol (25:24:1, Fluka) and 0.05g lysozyme (Sigma) were added. This was left to incubate for 10 minutes before being ribolysed twice (same procedure as RNA – see preceding section). The tube was centrifuged at 14000g for five minutes at 4ºC and the top aqueous layer was extracted and transferred to a new microcentrifuge tube. An equal volume of chloroform : isoamyl alcohol (24:1, Fluka) was added and shaken to form an emulsion. The tube was then centrifuged at 14000g for five minutes at 4ºC. The top aqueous layer was extracted and transferred to a new microcentrifuge tube where the nucleic acids were precipitated by adding two volumes of PEG solution (30% PEG 6000 [Fluka] and 1.6M NaCl [Sigma]). This was left for two hours on the bench before being centrifuged at 14000g for 10 minutes at room temperature. The supernatant was poured off and the pellet was washed with 200µl of 70% ethanol. The ethanol wash was poured off and the pellet was left to air dry for 10 minutes. The pellet was resuspended in 200µl of nuclease-free water and 3µl of RNaseA (Qiagen) was added and the tube was placed

in a water bath at 37 ºC for 30 minutes. The protocol was then repeated from the 0.5ml phenol : chloroform : isoamyl alcohol stage but the pellet was resuspended in 50µl of nuclease-free water and no RNase step was done. The DNA quality and purity was then assayed and stored at -80 ºC.

2.5.4 Assay Nuclease Quantity and Quality

RNA and DNA quantity and quality were assayed using a Nanodrop (ThermoScientific) and RNA quality was assayed further using a Bioanalyser (Agilent 2100 Bioanalyzer, Agilent Technologies).

2.5.4.1 Nanodrop

1.5µl of each sample was loaded on to a cleaned pedestal that had been freshly calibrated and this was drawn into the column and measured. Either the settings for RNA or DNA were used depending upon nucleic acid type.

2.5.4.2 Bioanalyzer

Agilent chips were loaded with RNA for sizing quantification and quality control on a Bioanalyser (Agilent 2100 Bioanalyser) following the standard protocol (Reagent kit guide, RNA 6000 NanoAssay, Nov 2003).

2.5.5 Labelling and Hybridisation

The protocol for RNA vs. DNA labelling and hybridisation using oligonucleotide arrays, from the Bacterial Microarray Group at St. George's, was followed. This work was conducted in the laboratories of the Bacterial Microarray Group at St. George's.

2.5.5.1 Microarray slides

The microarray slides were provided by the Pathogen Functional Genomics Resource Centre, part of the J. Craig Venter Institute. They are 70-mer aminosilane slide DNA microarrays. They have four technical replicates of 1948 oligonucleotides designed from the *S. mutans* complete genome sequence to represent all *S. mutans UA159*

genes. They have 96 grids on an aminosilane coating and have 500 *Arabidopsis thaliana* 70mer controls. They are *S. mutans* microarray version 1 slides.

2.5.5.2 Cy3 labelled DNA

For each microarray slide, following quantification of DNA concentration using the Nanodrop, 2µg of DNA and 1µl of Random primers (3µg/µl, Invitrogen) were mixed with enough nuclease-free water to make 41.5µl in an amber microcentrifuge tube (0.5ml volume, ideal for storing light sensitive material, Alpha Laboratories). The tube was heated at 95ºC for five minutes (on a Stuart block heater 5BH130DC) before being snap cooled on ice and briefly centrifuged (Eppendorf 5415D). Following centrifugation, 5µl of 10X React2 Buffer (REact®2, Invitrogen), 1µl dNTPs (5mM of each of dATP, dGTP and dTTP and 2mM of dCTP, Invitrogen), 1.5µl of Cy3 dCTP (GE Healthcare) and 1µl large fragment polymerase (Large Fragment Polymerase I, Invitrogen) were added to the tube. This was incubated in the dark at 37ºC for 90 minutes.

2.5.5.3 Cy5 labelled RNA

For each microarray slide, following quantification of RNA concentration using the Nanodrop, 5µg of RNA and 1µl of Random primers (3µg/µl) were mixed with enough nuclease-free water to make 11µl in an amber microcentrifuge tube (Alpha Laboratories). The tube was heated at 95ºC for five minutes before being snap cooled on ice and briefly centrifuged. Following centrifugation, 5µl of First Strand Buffer (Invitrogen), 2.5µl of DTT (100mM, Invitrogen), 2.3µl dNTPs (5mM of each of dATP, dGTP and dTTP and 2mM of dCTP), 1.7µl Cy5 dCTPs (GE Healthcare) and 2.5µl SuperscriptII Reverse Transcriptase (200U/µl, Invitrogen). This was incubated in the dark for 10 minutes followed by 42ºC in the dark for 90 minutes (PCR machine, MJ Research minicycler).

2.5.5.4 Slide prehybridisation

Prehybridisation solution (8.75ml 20X SSC [Sigma], 250µl 20%SDS [National Diagnostics], 5ml BSA [100mg/ml, Fraction V 96-99%, Sigma Aldrich] and 36ml ddH_2O) was made in a Coplin jar (Fisher Scientific) and incubated at 55ºC during the labelling reaction to equilibrate (Large incubator, Stuart Scientific). Once the solu-

tion was at 55 ºC the required number of microarray slides were incubated in the prehybridisation solution for 20 minutes. These were then placed in a slide staining rack (Raymond A. Lamb) and rinsed by oscillating the rack vigorously in a slide staining trough (Raymond A. Lamb) containing 400ml ddH_2O for one minute, followed by vigorous rinsing in 400ml of propan-2-ol (Sigma) for one minute. These slides were placed label down in falcon tubes and centrifuged at 1500rpm for five minutes (Fisher Scientific accuSpin1) to dry before being removed and stored in the dark in a dust free slide box until hybridisation (less than one hour).

2.5.5.5 Wash preparation

Wash A (20ml 20X SSC, 1ml 20%SDS and 379ml ddH_2O) was made and placed in a sealed bottle, along with a staining trough, at 55ºC.

2.5.5.6 Purification of Cy3 DNA/Cy5 labelled cDNA

The samples were cleaned following a Qiagen MinElute Purification protocol (Qiagen MinElute PCR Purification Kit, Qiagen). For each microarray slide the Cy3 DNA and Cy5 cDNA were combined in a single tube and 375µl Buffer PB (Qiagen) was added and mixed. This was applied to a chilled MinElute column in a collection tube (Qiagen) and centrifuged at 13000rpm for one minute at room temperature (Eppendorf, 5415D). The flow-through was discarded and the MinElute column was placed back in the same collection tube. 500µl Buffer PE (Qiagen) was added to the MinElute column before it was centrifuged again as before. Again the flow-through was discarded and 250µl of Buffer PE was added and centrifuged as before. Again the flow-through was discarded, the tube was centrifuged as before to remove any residual ethanol and the MinElute column was placed in a fresh 1.5ml amber tube (Alpha Laboratories). 27.5µl of nuclease-free water was added to the centre of the membrane and allowed to stand for one minute. This was then centrifuged as before and the elutant containing the purified DNA was assayed to verify fluorescent dye integration (1.5µl) using a Nanodrop (microarray settings) and the remainder was stored in the dark for hybridisation with the microarray.

2.5.5.7 Hybridise slide with Cy3 DNA and Cy5 labelled cDNA

For each microarray, each freshly prehybridised microarray slide was placed in a prehybridisation cassette (Corning, www.corning.com) and two 15µl aliquots of ddH_2O were added to the wells in the cassette. Three LifterSlips (22x22mm, VWR International, http://uk.vwr.com) were placed over the arrayed area of the slide ensuring the LifterSlip bars were face down. 75ml formamide-based hybridisation buffer (40% Formamide [Sigma], 5% Denhardt's solution [Sigma] 8X SSC, 1mM Sodium pyrophosphate [Sigma], 50mM Tris [pH 7.4, National Diagnostics] and 0.1% SDS [National Diagnostics]) was added to the purified Cy3 DNA/Cy5 cDNA and this mix was heated in a hot block (Stuart block heater, SBH130DC) for two minutes before being cooled gently to 70ºC (PCR machine set to 70ºC, MJ Research Minicycler). This mix was then carefully pipetted under the LifterSlips allowing the solution to be drawn completely across the array by capillary action. The hybridisation cassette was the sealed and placed in a water bath (Tupperware container filled with ddH_2O placed in 55ºC incubator, Stuart Scientific) in the dark for 16 to 20 hours.

2.5.5.8 Wash slide

Preheated Wash A was added to the preheated trough and the microarray slides were removed from the hybridisation cassettes and initially washed gently in the Wash A solution to gently remove the LifterSlips. They were then placed in the rack and washed vigorously for a further two minutes. The slides were then washed in a different trough containing 400ml of Wash B (1.2ml 20X SSC and 400ml of ddH_2O) for two minutes before being washed in a second trough of Wash B for a further two minutes. The slides were then dried in a centrifuge as before (1500rpm for five minutes, Fisher Scientific accuSpin1). The slides were then placed in a dust-free slide box in the dark ready for immediate scanning.

2.5.6 Scanning Microarray

Microarrays were scanned in an Affymetrix 428 Array Scanner. The Cy3 channel was scanned first using the highest gain setting that did not produce any white (saturated) readings to get the best dynamic range and then the same was done for the Cy5 channel.

2.5.7 Data Quantification Using Image Analysis

The scanned slides were annotated and quantified using BlueFuse for Microarrays 3.5(6446, BlueGnome Ltd.) and the PFGRC Ann file (annotation file recording Column Name, Description, Comments, Spot ID, Row, Column, Meta Row, Meta Column, Sub Row, Sub Column, Oligo ID, Sequence, GC%, Internal Repeat Score (IRS), Self-Annealing Score (SAS), Design Strain, Design Target, Common Name of Design Target, Gene Symbol and Strain).

2.5.8 Expression Analysis

Gene expression visualisation and analysis was conducted using GeneSpring (Agilent GeneSpring GX software).

2.6 Modelling Growth

The model was designed and executed using the software Wolfram Mathematica 6 (version 6.0.1.0). A novel *non-monotonic growth model* was developed by combining and extending the logistic growth model and the Lotka-Volterra predator-prey model.

2.6.1 Six steps of Modelling

Wanner et al. (2006) outline six steps for creating and using a mathematical model:

1. Identify the important variables and processes that are acting in the system
2. Represent the processes using mathematical expressions
3. Combine the mathematical expressions together in equations
4. Assign values to the parameters in the system
5. Solve the equations analytically or using numerical techniques
6. Run the model to output solutions of the properties of the system

These six steps form the basic outline of this Methods section.

2.6.1.1 Identify the important variables and processes that are acting in the system

S. mutans produces lactic acid from the metabolism of glucose. *V. dispar* metabolises lactic acid into weaker acids. The highly acidic medium as a result of the lactic acid limits growth and eventually kills both species but *S. mutans* has a higher tolerance for acidity than *V. dispar*. The microscopy studies conducted in Chapter 4 showed dense biofilms displaying little spatial structure, thus spatial structure was not included in the model and hence diffusion was not included in the model. Excluding spatial structure and diffusion makes the form of the model instantly simpler. The key processes are the exponential growth of both species, limited by the presence of lactic acid, the production and metabolism of lactic acid, and eventual death caused by the accumulation of lactic acid. Thus the key factors of the model are the numbers of *S. mutans* and *V. dispar*, the time in days, the concentration of lactic acid in mol/l, the concentrations of lactic acid in mol/l where the growth of *S. mutans* and *V. dispar* cease and beyond which growth is negative, growth rates of *S. mutans* and *V. dispar* (including possible differences in growth rates for *V. dispar* in the presence and absence of lactic acid) and finally the amounts of lactic acid produced by *S. mutans* and consumed by *V. dispar*.

2.6.1.2 and 6.2.1.3 Represent the processes using mathematical expressions and Combine the mathematical expressions together in equations

These steps have been combined for the purpose of illustrating the non-monotonic growth model.

Non-monotonic growth model

$$\frac{ds}{dt} = rs(1-\frac{h}{\lambda})$$

$$\frac{dv}{dt} = (\alpha + \omega h)v(1-\frac{h}{\Psi})$$

$$\frac{dh}{dt} = \beta s - \phi v$$

$$\frac{dq}{dt} = ws(1-\frac{h}{y})$$

s is the number of *S. mutans*,

v is the number of *V. dispar*,

q is the number of a competing species,

t is the time in days,

h is the concentration of lactic acid in mol/l,

λ is the concentration of lactic acid in mol/l where growth of *S. mutans* ceases and beyond which is negative,

Ψ is the concentration of lactic acid in mol/l where growth of *V. dispar* ceases and beyond which is negative,

y is the concentration of lactic acid in mol/l where growth of a competitor ceases and beyond which is negative,

r is the growth rate of *S. mutans*,

α is the growth rate of *V. dispar* excluding any increased growth rate from the presence of lactic acid,

ω is a factor that captures the increase in growth rate of *V. dispar* in the presence of lactic acid,

w is the growth rate of a competitor,

β is the amount of lactic acid produced by *S. mutans*,

φ is the amount of lactic acid consumed by *V. dispar*.

Note the extra equation added to model the addition of a hypothetical competing species (q) that neither produces nor metabolises lactic acid but that is susceptible to pH (y).

This is a deterministic, single-scale, qualitative, simple, multiple species, non-dimensional, dynamical, single process model.

2.6.1.4 Assign values to the parameters in the system

While β and φ were both estimated, other parameters were set or measured during the experiments (see Chapter 3). Table 2.A shows the parameters that were assigned initially.

Table 2.A Initial parameters of non-monotonic growth model

Parameter	Assigned Value
s is the number of *S. mutans*	10^7
v is the number of *V. dispar*	10^7
t is the time in days	14
h is the concentration of lactic acid in mol/l	0
λ is the concentration of lactic acid in mol/l where growth of *S. mutans* ceases and beyond which growth is negative	$1.3x10^{-5}$
Ψ is the concentration of lactic acid in mol/l where growth of *V. dispar* ceases and beyond which growth is negative	$5.0x10^{-6}$
r is the growth rate of *S. mutans*	$2x10^3$
α is the growth rate of *V. dispar* excluding any increased growth rate from the presence of lactic acid	$2x10^2$

Lactic acid concentration is used as a proxy for H+ to simplify the model but the critical thresholds, determined by measuring pH in experiments, were calculated correctly, by taking into account the concentration of associated and undissociated lactic acid that equates to the critical pH thresholds measured. An example calculation showing the concentration of lactic acid when the pH was 5.0 is shown below.

Lactic acid is a weak acid and does not dissociate completely in water. The acid dissociation constant K_a is the equilibrium constant of the dissociation reaction and thus is a measure of the strength of an acid in solution. For lactic acid it is 1.39×10^{-4} (3 significant figures, from $pK_a = -\log_{10} K_a$, Speight 2005 [Lange's Handbook of Chemistry 16th Edition]).

$$CH3CH(OH)COOH \rightleftharpoons H^+ + CH3CH(OH)COO^-$$

If the pH is 5.00 then the concentration of H^+ is 1.00×10^{-5}M

pH	=	5.00	=	$-\log [H^+]$
$[H^+]$	=	inv log (-5.00) =		$10^{-5.00}$
$[H^+]$	=	1.00×10^{-5} M		

Then the concentration of lactic acid (associated and dissociated) is 1.07×10^{-5}M

K_a	=	$[H^+][B^-]/[HB]$
K_a	=	$[H^+]^2/(C-[H^+])$
C	=	$([H^+]^2 + K_a[H^+])/K_a$
C	=	$((1.00 \times 10^{-5})^2 + (1.39 \times 10^{-4} \times 1.00 \times 10^{-5})) / (1.39 \times 10^{-4})$
C	=	1.07×10^{-5}

2.6.1.5 Solve the equations analytically or using numerical techniques

This was done using Mathematica 6 (version 6.0.1.0). A Mathematica Demonstrations project that modelled the Lotka Volterra equations (Weisstein 2010) was used as a base for the initial code of the non-monotonic growth model.

2.6.1.6 Run the model to output solutions of the properties of the system

This was done using Mathematica 6 (version 6.0.1.0) with the calculated parameters and with changed parameters to employ the model in a totally non-dimensional way, thus looking at the shape and form of the output rather than the precise numbers.

2.6.1.7 Use the model to make testable hypotheses

A further step was added which was to use the model to make testable hypotheses. This was primarily with regard to test theoretical ways to modulate the system beneficially and to model the effect of a hypothetical competitor that neither produces nor metabolises lactic acid but that is susceptible to pH having a critical pH beyond which growth is negative. Given additional time, this would have been extended by validating these hypotheses experimentally (that is adding a competitive third species in to the biofilm inoculum), re-evaluating the theory based upon the results, and feeding the new theory back in to the model.

When applying this model, it became evident that it was not sufficient to describe the behaviour of the system when using the measured variables, however treating it as an essentially dimensionless model, where it is the form and shape of the relationships that are important, it reproduced the basic patterns of the relationships and was useful in predicting as yet untested dynamics of the system. However, an alternative model form was trialled as well to capture the shape of the model.

Searching the literature identified the *incipient growth processes with competing mechanisms* model (Normand et al. 2010) that has the kind of shape observed in the experimental chapter. The form of this model allows the shape to be adjusted so that it could fit any five points that follow the basic growth shape observed in the experimental chapter. The model however has five parameters so fitting this model to five points is termed ‘over-fitting’. Also, it requires the input of key switch points (characteristic time scales, tc1 and tc2, two of the parameters) which ideally should arise as an emergent property of the model. Thus it is not employed to replace the non-monotonic growth model but rather to show how changing the structure of the model may be useful in capturing the properties of the system. The form of each of the growth and decay components in this model are very similar to the logistic growth model.

Incipient growth processes model

Two versions of this model were trialled; using bases 2 and e, base 2 because bacteria grow by binary fission and base e as e^x equals its own derivative and consequently is mathematically convenient.

$$P = P_0 2^{((\frac{t}{t_{c1}})^{m_1} - (\frac{t}{t_{c2}})^{m_2})}$$

$$P = P_0 e^{((\frac{t}{t_{c1}})^{m_1} - (\frac{t}{t_{c2}})^{m_2})}$$

P_0 is the initial population,

t_{c1} is the characteristic timescale for the growth process,

t_{c2} is the characteristic timescale for the death process,

m_1 is the rate parameter of the growth process,

m_2 is the rate parameter of the death process.

3 Growth Studies

3.1 Introduction

This study investigated how *S. mutans* and *V. dispar* grew in the presence of each other, and whether there were differences in growth rates, maximum population sizes and survival. This study also investigated whether the presence of the other species affected the environment they each could live in and how they changed their environment when grown separately and alone. These growth studies are modelled in Chapter 6. Some of the biofilms grown in these studies were investigated morphologically and these investigations are reported in Chapter 4. The data was collected to identify any social interactions occurring but also in a manner that was useful for providing a quantitative explanation of the processes occurring, as reported in Chapter 6.

Key factors relating to bacterial fitness are growth, population size and survival, so the effects on these of the presence of the other species were investigated. A range of single and dual species growth experiments were conducted to investigate the effect of the phenotype of the bacteria (planktonic/biofilm), the effect of pH and buffering, the effect of differences in relative population size and the effect of the amount of sugar (D-glucose) available.

These experiments were conducted to determine if the presence of the other species did have effects on key aspects of bacterial fitness and to determine if these effects were direct or indirect (an indirect effect would be if they change the environment and this in turn affects fitness) with the ultimate aim of investigating the nature of relationships between unrelated species.

3.1.1 Aims

(i) to characterise the planktonic growth, maintenance and decline of *S. mutans* and *V. dispar* in isolation and combination.

(ii) to investigate the effects that these two species have on each other when grown as a dual-species culture.

(iii) to investigate the effect the different planktonic cultures have on the pH of their environment.

(iv) to investigate the effects that buffering the medium to acidic, neutral and alkaline pH has on the growth of, and interactions between, the species.

(v) to characterise the formation, growth, maintenance and decline of biofilms comprising these two species in isolation and combination.

(vi) to investigate the effects these two species have on each other when grown as a dual-species biofilm.

(vii) to investigate the effect the biofilms have on the pH of the surrounding environment.

3.2 Materials and Methods

The methods are outlined in Chapter 2.

3.3 Results

3.3.1 Development of Methodology

Preliminary experiments were conducted to develop and refine the methods employed to grow and assay biofilms. These studies are reported in Appendix 2.

3.3.2 Investigation of Single and Dual Species Planktonic Cultures

Having developed the sampling regime, the interactions of *S. mutans* and *V. dispar* were investigated in unbuffered and buffered liquid culture.

3.3.2.1 Investigating the interactions between *S. mutans* and *V. dispar* in planktonic culture

The two species were grown together and separately for 14 days. Total viable counts, pH and vitality were measured and are presented here.

3.3.2.1.1 Planktonic culture - total viable counts

S. mutans and *V. dispar* in both single species cultures and dual-species cultures grew rapidly and then gradually decreased in numbers, except for *V. dispar* which showed a different pattern of growth in the later stages of the experiment (see Figure 3.A). *S. mutans* displays similar curves for single and dual species but better survival in dual species biofilms while *V. dispar* growing in dual species biofilms displays a trough in numbers at the seven day time point but appears to adapt to the low pH and start growing again using the rich energy source of lactic acid (seeFigure 3.A).

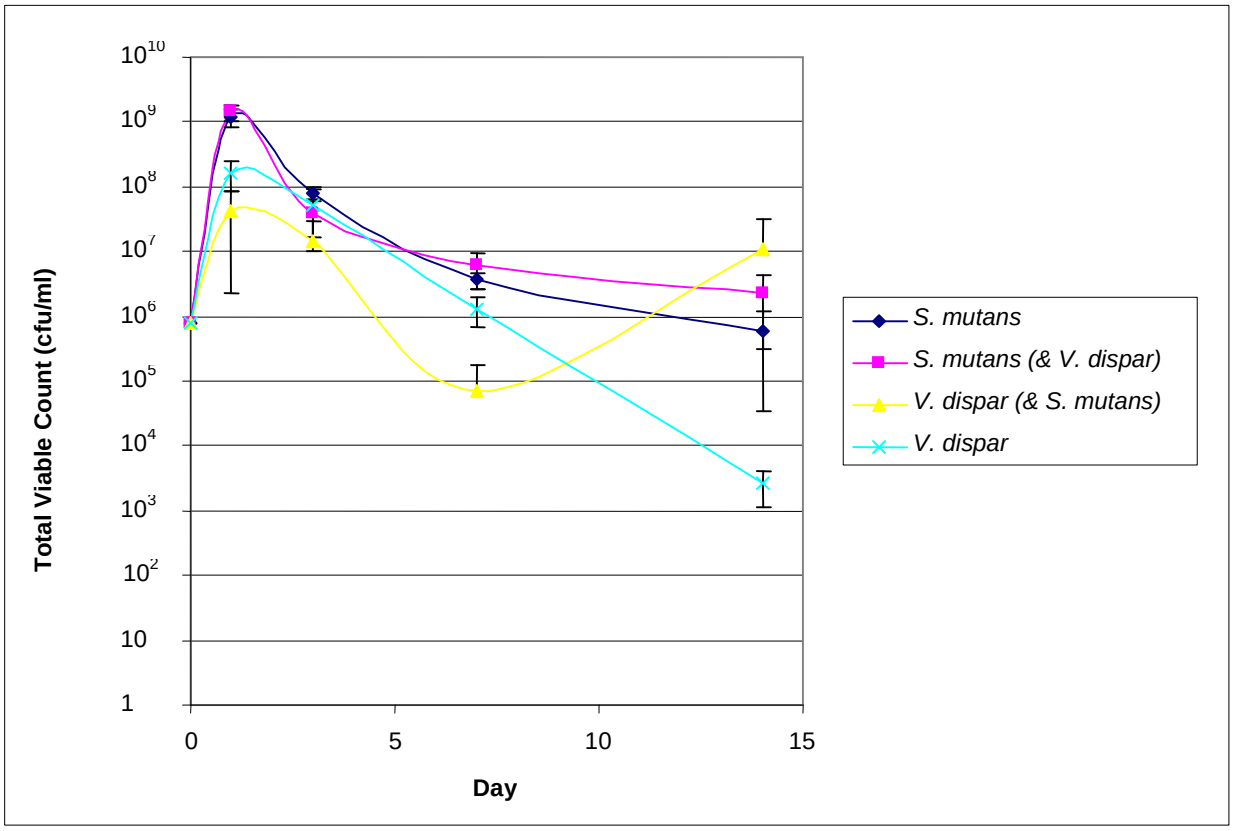


Figure 3.A. Total viable counts of both species of bacteria growing together and alone in unbuffered medium over the four time points. Arithmetic means are plotted with error bars showing one standard deviation. Single species *S. mutans* counts are plotted in dark blue. Single species *V. dispar* counts are plotted in light blue. The counts of dual-species cultures are plotted in pink and yellow with pink showing the means of the counts of *S. mutans* and yellow showing the means of the counts of *V. dispar*.

A general linear model (a statistical linear model that incorporates a number of statistical models including ANOVA, MANCOVA and multiple regression) was constructed to analyse total viable counts of *S. mutans*. The length of broth growth was highly significant with every day having different means that decreased throughout the experiment ($F=348.217$, d.f.=4, $P<0.001$). The covariate pH was significant ($F=8.403$, d.f.=1, $P=0.006$) and the interaction of broth growth and single/dual species broth was highly significant ($F=5.337$, d.f.=4, $P=0.002$). The model itself was highly significant ($F=5256.544$, d.f.=9, $p<0.001$) and explained 99.9% of the variation in *S. mutans* total viable counts (adjusted R^2). The only significant difference between single and dual species *S. mutans* was dual species *S.*

mutans had significantly lower counts at the 3 day time-point than single species *S. mutans* ($p<0.05$).

A general linear model was constructed to analyse total viable counts of *V. dispar*. The length of broth growth was highly significant with every day having different means that decreased throughout the experiment ($F=36.530$, d.f.=3, $P<0.001$). Whether *V. dispar* was growing as single or dual species broths was highly significant with dual species broths having higher counts ($F=18.238$, d.f.=1, $P<0.001$). The covariate pH was highly significant ($F=20.428$, d.f.=1, $P<0.001$). The model itself was highly significant ($F=24.371$, d.f.=5, $p<0.001$) and explained 71.3% of the variation in *V. dispar* total viable counts (adjusted R^2).

3.3.2.1.2 Planktonic culture - pH

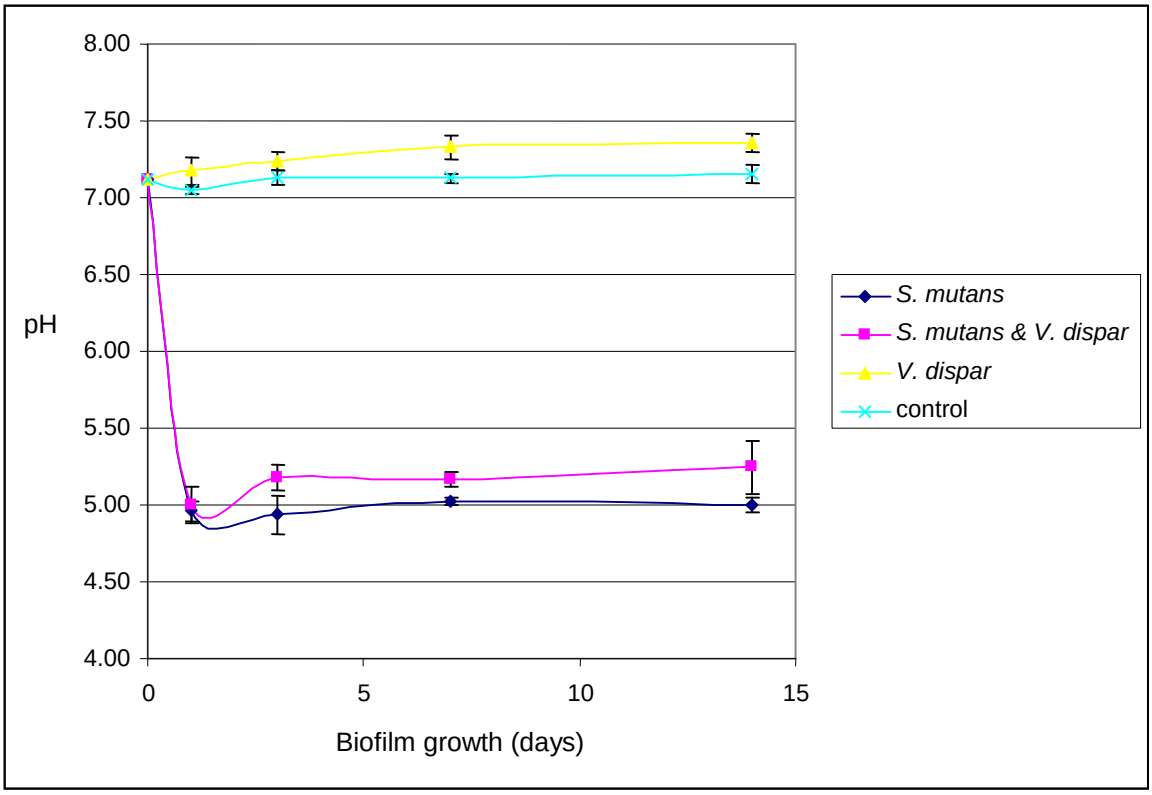


Figure 3.B. pH of single and dual species broth cultures.
Means are plotted with error bars showing one standard deviation.

After three days of growth dual species cultures were less acidic than *S. mutans* single species biofilms (see Figure 3.B). *V. dispar* cultures increased slightly in pH because of the alkaline end-products of peptone breakdown (Doel et al. 2005, Wilson 2005).

3.3.2.1.3 Planktonic culture - vitality

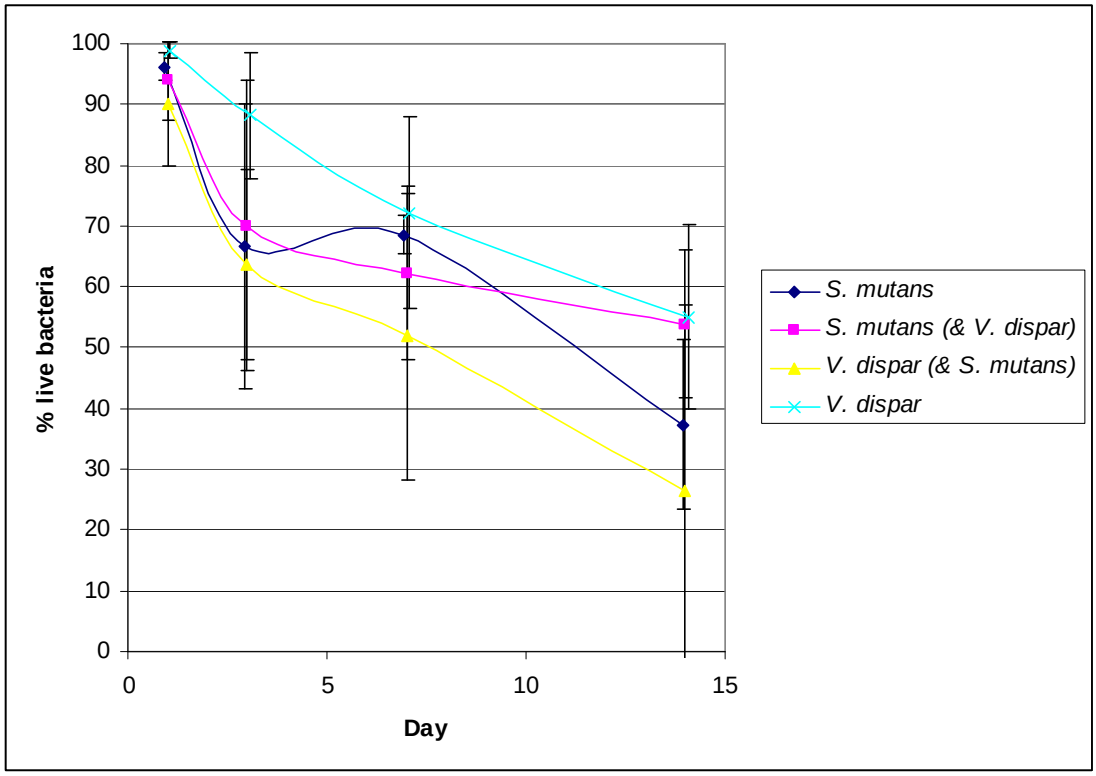


Figure 3.C. Vitality of both species of bacteria growing together and alone in unbuffered medium over the four time points. The pink line and pink boxes show the percentage of live *S. mutans* bacteria when *S. mutans* was grown with *V.dispar*, and the yellow line with yellow triangles show the percentage of live *V. dispar* bacteria when *V. dispar* was grown with *S. mutans*. Mean percentages are plotted with error bars showing one standard deviation.

The vitality of all of the bacteria declined throughout the experiment but the data was noisy (see Figure 3.C). There was a non-significantly greater percentage of dead *V. dispar* in dual species cultures presumably due to toxic stress caused by lactic acid.

3.3.2.1.4 Planktonic culture - summary

Table 3.A. Summary of growth in unbuffered planktonic culture.

	Final pH	σ of Final pH	dN/dt	K (cfu)	σ of K (cfu)
S. mutans	5.00	0.05	1529	1.2×10^9	3.7×10^8
S. mutans & *V. dispar*	5.24	0.18	1786	1.4×10^9	4.2×10^8
S. mutans & ***V. dispar***	5.24	0.18	52	4.3×10^7	4.0×10^7
V. dispar	7.36	0.06	203	1.6×10^8	7.9×10^7

Table 3.A provides a summary of pH for the 3 biofilm types and rates of growth dN/dt [change in population size (dN) with respect to change in time (dt)] carrying capacities (K [maximum population]) for the 2 species either growing separately or together (values are shown for the species in bold type in the left column). σ represents the standard deviation and is shown for Final pH and K. Maximum population size was reached for both species growing separately or alone after one day of growth.

V. dispar grew faster (higher dN/dt's – did not measure r as delta t covered a broader time period than the period of exponential growth) and reached a higher carrying capacity (higher Ks) in single species cultures but dual species *V. dispar* cultures had significantly higher total viable counts for the experiment as a whole. dN/dt is a rate of growth but is not r as r is by definition the growth rate during exponential growth. If delta t is a period of time when the bacteria are displaying unlimited exponential growth, then dN/dt is equivalent to r. *S. mutans* was very similar in single and dual species cultures. This suggests *S. mutans* is not gaining a discernable benefit from *V. dispar* removing some of the lactic acid and thus increasing the pH.

3.3.2.2 Investigating the interaction between *S. mutans* and *V. dispar* in buffered planktonic culture

3.3.2.2.1 Buffered planktonic culture - total viable counts

This experiment investigated the interaction of these two species in buffered planktonic culture. Figure 3.D shows total viable counts when the planktonic culture was buffered to pHs of 5.1, 6.3, 7.2 and 8.1. Total viable counts are shown for each

species growing as single or dual-species planktonic cultures. *S. mutans* grew to the highest total viable counts at neutral pH (pH = 7.2). *S. mutans* could grow at all pHs but died off initially in the alkaline buffer (pH = 8.1) before adapting and growing. *V. dispar* died when buffered with acetate (pH = 5.1), grew slowly in alkaline conditions (pH = 8.1), grew reasonably at neutral pH (pH = 7.2) and grew to the highest total viable counts in slightly acidic conditions (pH = 6.3). The different buffers worked well as buffers as there was only an average pH change of -0.10±0.14 and a range of pH changes of -0.27 to 0.17.

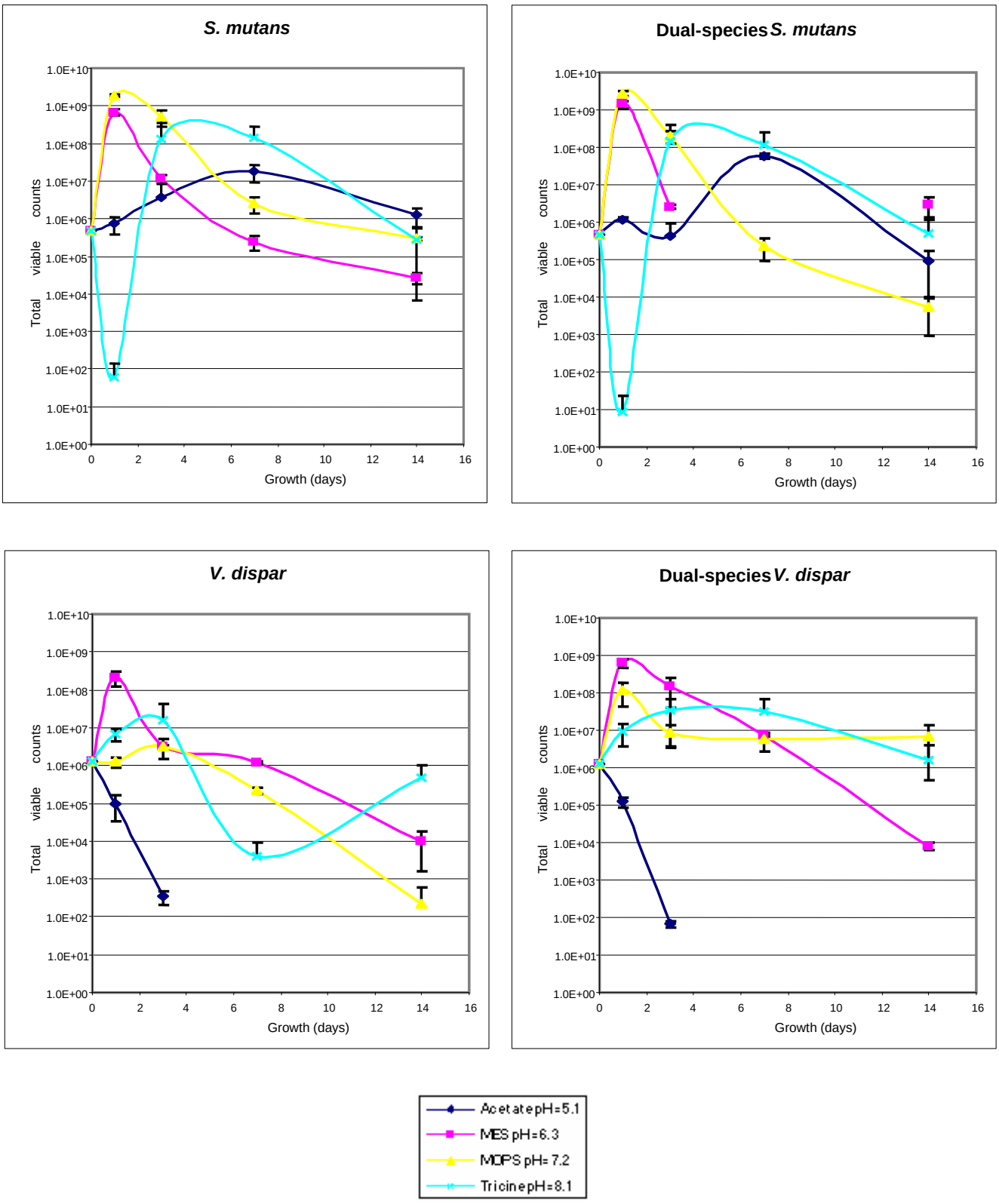


Figure 3.D Total viable counts in buffered planktonic culture

The total viable counts of *S. mutans*, dual-species *S. mutans*, *V. dispar* and dual-species *V. dispar* when grown as planktonic cultures in media buffered to pH 5.1 (Acetate buffer), pH 6.3 (MES buffer), pH 7.2 (MOPS buffer) and pH 8.1 (Tricine buffer). Error bars show one standard deviation.

A general linear model was constructed to analyse total viable counts of *S. mutans* growing in buffered liquid medium. The type of buffering was highly significant (F=17.796, d.f.=1, P<0.001). The length of broth growth was highly significant (F=6.192, d.f.=3, P=0.001) and the interaction of buffering and length of broth growth was highly significant (F=16.992, d.f.=9, P<0.001). The model itself was highly significant (F=1910.855, d.f.=9, p<0.001) and explained 70.4% of the variation in *S. mutans* total viable counts (adjusted R^2). Whether *S. mutans* was growing in a single or dual species in buffered liquid medium was not significant.

A general linear model was constructed to analyse total viable counts of *V. dispar* growing in buffered liquid medium. The type of buffering was highly significant (F=59.587, d.f.=3, P<0.001). The length of broth growth was highly significant (F=43.359, d.f.=3, P<0.001). Whether *V. dispar* was growing as a single or dual species broth was highly significant with dual species having higher counts (F=22.775, d.f.=1, P<0.001). The interaction of buffering and length of broth growth was significant (F=2.480, d.f.=9, P=0.015). The interaction of buffering and whether *V. dispar* was growing as a single or dual species broth was significant (F=3.294, d.f.=3, P=0.025) with fourteen-day-old dual-species biofilms buffered to 7.2 (MOPS buffer) having significantly higher *V. dispar* total viable counts than the comparable single-species biofilms. The model itself was highly significant (F=19.148, d.f.=19, p<0.001) and explained 78.4% of the variation in *V. dispar* total viable counts (adjusted R^2).

3.3.2.2.2 Buffered planktonic culture - summary

This series of experiments demonstrated the pH tolerance of the two species and that *V. dispar* could thrive in the presence of *S. mutans* if lactic acid was prevented from accumulating. *S. mutans* also benefited from the buffering reaching higher carrying capacities in medium buffered to a neutral pH than in the unbuffered medium in the previous experiment. While the difference was not significant using the general linear model, *S. mutans* still had higher carrying capacities growing in the presence of *V. dispar* for all pHs.

If this difference is investigated by comparing means of single and dual species buffered averages of max K (see Table 3.B), they are not significantly different except for dual species Acetate (pH = 5.1) which had a higher mean K than single species Acetate (p=0.045, Dunnett's T3 post hoc test on oneway ANOVA, F=54.599, d.f.=7, p<0.001, other means were also different when comparing between the different buffers).

Table 3.B. Summary of growth in buffered planktonic culture.

Biofilm	Buffer	dN/dt	Max dN/dt (days)	K (cfu)	σ of K (cfu)	max K (days)
S. mutans	Acetate	2	1 to 3	$1.8x10^{7}$	$8.4 x10^{6}$	7
	MES	1417	0 to 1	$6.7 x10^{8}$	$1.3 x10^{8}$	1
	MOPS	4112	0 to 1	$1.9 x10^{9}$	$1.6 x10^{8}$	1
	Tricine	1140000	1 to 3	$1.4 x10^{8}$	$1.5 x10^{8}$	7
S. mutans & *V. dispar*	Acetate	36	3 to 7	$6.0 x10^{7}$	$9.0 x10^{6}$	7
	MES	3031	0 to 1	$1.4 x10^{9}$	$3.1 x10^{8}$	1
	MOPS	5832	0 to 1	$2.7 x10^{9}$	$4.9 x10^{8}$	1
	Tricine	9115000	1 to 3	$1.5 x10^{8}$	$2.6 x10^{8}$	3
S. mutans & ***V. dispar***	Acetate	0	0	$1.3 x10^{6}$	0	0
	MES	486	0 to 1	$6.3 x10^{8}$	$1.8 x10^{8}$	1
	MOPS	88	0 to 1	$1.2 x10^{8}$	$7.3 x10^{7}$	1
	Tricine	6	0 to 1	$3.5 x10^{7}$	$3.1 x10^{7}$	3
V. dispar	Acetate	0	0	$1.3 x10^{6}$	0	0
	MES	159	0 to 1	$2.1 x10^{8}$	$8.8 x10^{7}$	1
	MOPS	1	1 to 3	$3.3 x10^{6}$	$1.8 x10^{6}$	3
	Tricine	4	0 to 1	$1.5 x10^{7}$	$2.6 x10^{7}$	3

Table 3.B provides a summary of rates of growth (dN/dt) Maximum population (carrying capacities, K) for the 2 species either growing separately or together (values are shown for the species in bold type in the left column). σ represents the standard deviation and is shown for K.

The large values of dN/dt in single and dual species *S. mutans* grown in Tricine equate to doubling times (t_D, generation time) of 160 and 130 minutes (if dN/dt is substituted in as r, assuming t covers an exponential period of growth only).

$$t_D = \frac{dt}{\log_2 r}$$

These are reasonable estimates of doubling time as the doubling time of *S. mutans* (under acid stress as opposed to alkaline stress) at pH 5 is 164 minutes (at pH 7.5 it is

70 minutes, Korithoski et al. 2007). The reason the values for dN/dt are much lower than this for the other treatments is that because of the sampling effort involved, it is not feasible to sample growth while the treatments are still in the exponential phase thus dN/dt is not equivalent to r.

3.3.3 Investigation of Single and Dual Species Biofilms

Single and dual species biofilms were grown together in four repeated tripled experiments (*S. mutans*, *S. mutans* + *V. dispar*, and *V. dispar*). Four experiments were conducted growing three types of biofilms and sampling four replicates for each of four time points (one, three, seven, and fourteen days; as outlined in Figure 3.C in Methods).

3.3.3.1 Influence of initial inocula ratios on dual species biofilm growth (10 : 1 *S. mutans* : *V. dispar*)

This experiment is referred to as the 10 *S. mutans* to 1 *V. dispar* biofilm experiment hereafter.

This experiment was investigating a situation similar to what would occur in a dental plaque biofilm where *V. dispar* invades an already established *S. mutans* biofilm. To investigate this consistently experimentally, both species were inoculated on to the biofilm at the same time but 10 times as many *S. mutans* were in the inoculate as *V. dispar*.

3.3.3.1.1 Total viable counts of the two species in the 10 *S. mutans* to 1 *V. dispar* biofilm experiment

The bacteria grew after inoculation to form biofilms which were assayed from one day of growth. Figure 3.E shows the results of four independent cohorts, with four replicates of each of the three biofilm types. Total viable counts of each species were analysed using general linear models. *S. mutans* grew rapidly for the first day and then continued to grow, but slower, up to three days before declining in numbers in both single and dual species biofilms. *V. dispar* grew rapidly for one day before declining in numbers. *V. dispar* declined catastrophically in numbers after seven days in dual species biofilms. Figure 3.F is shown in the same page to demonstrate

how pH changes as the biofilms change. The pH decreased in single and dual species biofilms containing *S. mutans* and rose slightly in single species *V. dispar* biofilms.

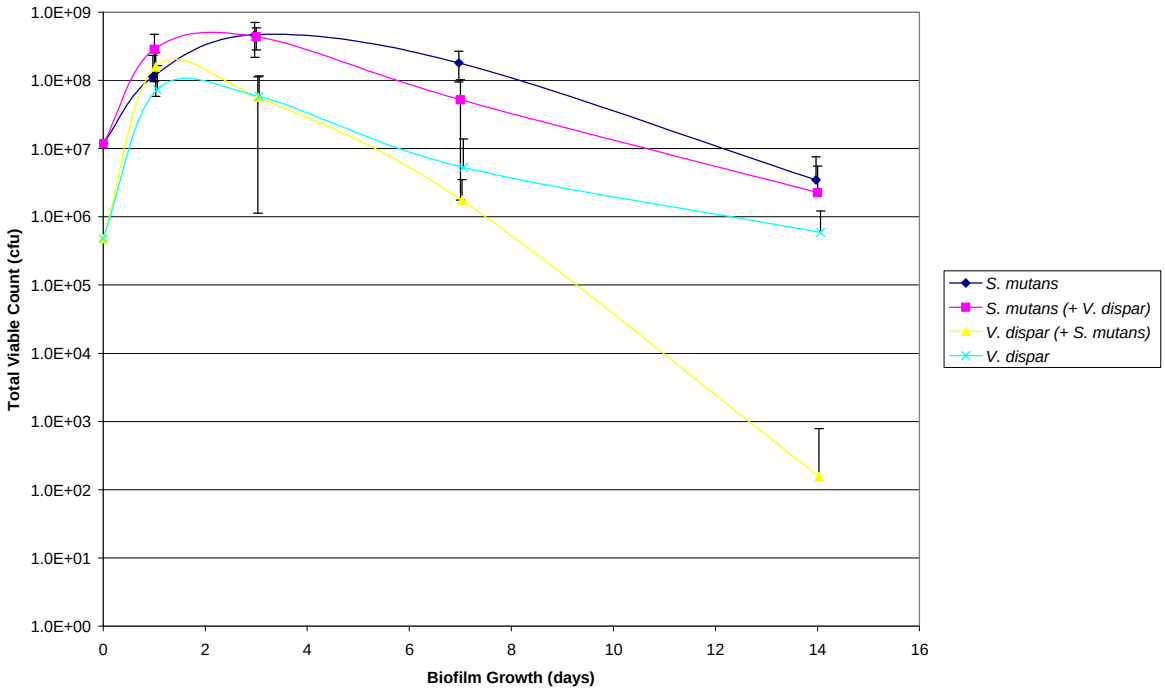


Figure 3.E. Total viable counts of both species of bacteria growing together and alone over the four time points in the 10 *S. mutans* to 1 *V. dispar* experiment. Note that all but one of the dual species biofilms had no viable *V. dispar* at 14 days (can not display zero on a log scale). Error bars show one standard deviation.

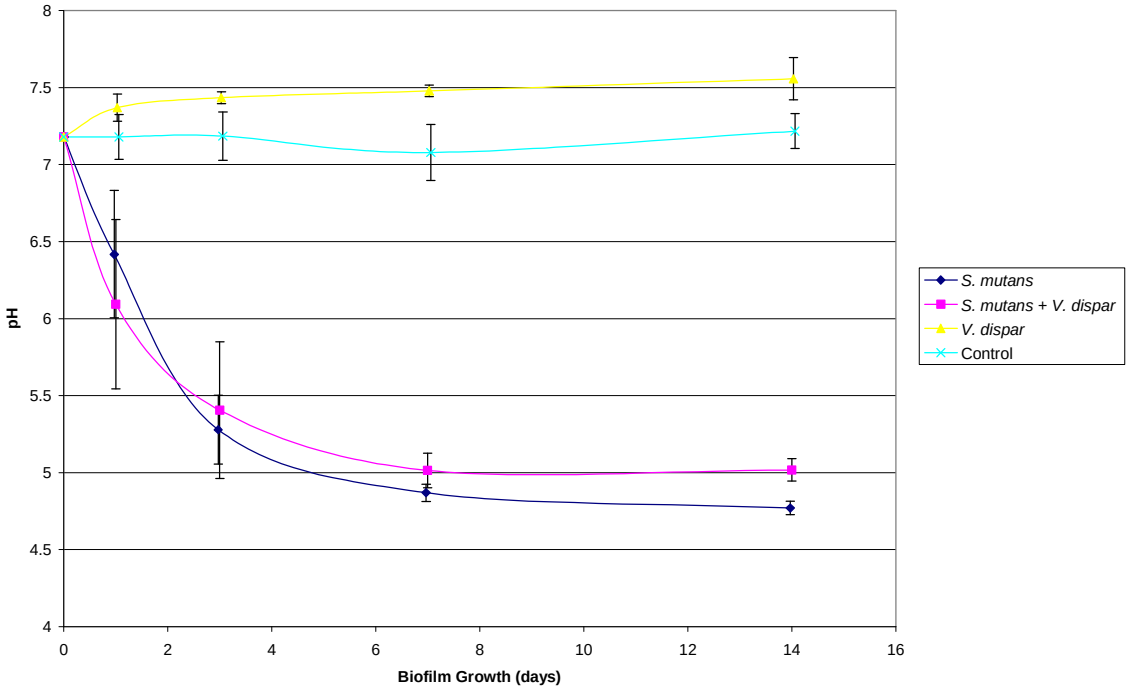


Figure 3.F. pH of the different biofilms in the 10 *S. mutans* to 1 *V. dispar* experiment at the four time points. Error bars show one standard deviation.

A general linear model was constructed to analyse total viable counts of *S. mutans.* The interaction of biofilm growth and whether *S. mutans* was growing as a single or dual species biofilm was highly significant with 7 day dual species biofilms having lower total viable counts (F=3.482, d.f.=4, P=0.010, 1 and 3 day dual species biofilms had higher counts but not significantly so). The model itself was highly significant (F=21.794, d.f.=19, p<0.001) and explained 76.3% of the variation in *S. mutans* total viable counts (adjusted R^2).

A general linear model was constructed to analyse total viable counts of *V. dispar.* The interaction between length of growth and whether *V. dispar* was growing as a single or dual species biofilm was highly significant with dual species biofilms having higher viable counts after 1 day but lower counts after 14 days (F=23.417, d.f.=4, P<0.001). The interaction of cohort, biofilm growth and whether *V. dispar* was growing as a single or dual species biofilm was highly significant (F=4.073, d.f.=21, P<0.001). The model itself was highly significant (F=17.692, d.f.=31, p<0.001) and explained 80.3% of the variation in *V. dispar* total viable counts (adjusted R^2).

The next two general linear models investigated the untransformed (as comparing covariance) total viable counts of both species within the dual species biofilms, and the covariance with the total viable count for the other species. The general linear model for the total viable count of *S. mutans* fitted the data well (adjusted R^2 = 0.822) and was highly significant (F=19.143, d.f.=16, P<0.001). The covariance with the total viable counts of *V. dispar* was significant (F=4.378, d.f.=1, P=0.042). This means that *S. mutans* total viable counts covaried with *V. dispar* total viable counts The general linear model for the total viable count of *V. dispar* fitted the data well (adjusted R^2 = 0.763) and was highly significant (F=13.711, d.f.=16, P<0.001). The covariance with the total viable counts of *S. mutans* was of course the same as the previous model (as it is the same calculation) and was significant (F=4.378, d.f.=1, P=0.042).

Given that *S. mutans* and *V. dispar* total viable counts co-vary, and whether it was a single or dual species biofilm was a highly significant factor (p=0.01) in the general linear model for *S. mutans*, and a very highly significant factor (p<0.001) in the

general linear model of *V. dispar*, it is likely that *S. mutans* total viable counts may modulate *V. dispar* total viable counts more than *V. dispar* total viable counts modulate *S. mutans* total viable counts.

The general linear models investigated the whole time course of the experiment and thus used Log_2 transformed data to not violate the assumptions of the models. Within a particular time point it is possible to compare the differences between untransformed data of the different treatments and these results are presented below.

The means of total viable counts after one day were significantly different (oneway ANOVA, F=7.927, d.f.=3, P<0.001), with *S. mutans* growing in dual species biofilms having higher total viable counts than *S. mutans* growing in single species biofilms (P=0.030) and higher total viable counts than *V. dispar* growing in single species (P=0.003) but not dual species (P=0.137) biofilms. *S. mutans* growing in single species biofilms had no significant difference in total viable counts to *V. dispar* grown in single or dual species biofilms (P=0.858, P=0.828). There was no significant difference in total viable counts between *V. dispar* grown in single and dual species biofilms (P=0.097).

The means of total viable counts after three days of growth were significantly different (oneway ANOVA, F=36.100, d.f.=3, P<0.001), with *S. mutans* growing in single and dual species biofilms having no significant difference in total viable counts (P=0.999) but both single and dual species *S. mutans* biofilms having significantly higher total viable counts than both single and dual species *V. dispar* total viable counts (P<0.001, P<0.001, P<0.001, P<0.001). There was no significant difference in total viable counts between *V. dispar* grown in single and dual species biofilms (P=1.0).

The means of total viable counts after seven days of growth were significantly different (oneway ANOVA, F=44.881, d.f.=3, P<0.001), with *S. mutans* growing in single species biofilms having significantly higher total viable counts than dual species *S. mutans* total viable counts (P<0.001) and both single and dual species *V. dispar* total viable counts (P<0.001, P<0.001). *S. mutans* grown in dual species biofilms had significantly higher total viable counts than *V. dispar* grown in single

and dual species biofilms (P=0.012, P=0.007). There was no significant difference between *V. dispar* grown in single and dual species biofilms (P=0.508).

The means of total viable counts after fourteen days of growth were significantly different (oneway ANOVA, F=5.777, d.f.=3, P=0.002), with *S. mutans* growing in single species biofilms having significantly higher total viable counts than dual species *V. dispar* total viable counts (P=0.023) but not single species *V. dispar* total viable counts (P=0.074). There was no significant difference between *S. mutans* grown in single and dual species biofilms (P=0.923). *S. mutans* grown in dual species biofilms had no significant difference in total viable counts to *V. dispar* grown in single (P=0.289) or dual (P=0.075) species biofilms. *V. dispar* grown in dual species biofilms had significantly lower total viable counts than *V. dispar* grown in single species biofilms (P=0.010).

In summary, after one day of biofilm growth, *S. mutans* growing in dual species biofilms had significantly higher total viable counts than when grown in single species biofilms. *V. dispar* also had higher total viable counts when grown in dual species biofilms although this difference was not significant (but it was close to being so, P=0.097). After three days of growth there were no differences between total viable counts of single and dual species biofilms for either species. After seven days, single species *S. mutans* biofilms had higher total viable counts than dual species *S. mutans* biofilms but there was no significant difference between single and dual species *V. dispar* biofilms. After fourteen days there was no significant difference between *S. mutans* single and dual species total viable counts but total viable counts of dual species *V. dispar* biofilms had collapsed and this difference was significantly different to single species *V. dispar* biofilms (see Figure 3.E).

3.3.3.1.2 pH of the environment (medium) in the 10 *S. mutans* to 1 *V. dispar* biofilm experiment

The pH of the medium was measured in triplicate for each replicate, at each time-point of each biofilm type. The pH of the biofilms was investigated using a general linear model using pH as the dependent factor and cohort, biofilm growth (days) and type of biofilm (*S. mutans*, *S. mutans* + *V. dispar*, *V. dispar*, control) as the factors. This model fit the data very well (adjusted R^2 = 0.982) and was highly significant

(F=175.969, d.f.=62, P<0.001). All factors and interactions of factors were highly significant ([cohort F=27.803, d.f.=3, P<0.001], [biofilm growth F=146.047, d.f.=3, P<0.001], [type of biofilm F=2575.399, d.f.=3, P<0.001], [cohort*day F=4.159, d.f.=9, P<0.001], [cohort*type of biofilm F=7.724, d.f.=9, P<0.001], [biofilm growth*type of biofilm F=86.542, d.f.=9, P<0.001], [cohort*biofilm growth*type of biofilm F=3.029, d.f.=26, P<0.001]). This means that the experiment, the amount of biofilm growth, the type of biofilm and the way these factors interacted were all important in determining the pH of the biofilm and they could together explain 98.2% of the variation in pH.

The pH decreased rapidly in all biofilms containing *S. mutans* although dual species biofilms decreased less (see Figure 3.F – shown in previous section on same page as Figure 3.E to facilitate comparison). The pH of *V. dispar* single species biofilms rose slightly. Single species *S. mutans* biofilms stabilised at a pH of 4.77±0.04 compared with 5.02±0.07 in dual species biofilms (fourteen day, average ± standard deviation, see Table 3.A) and this difference was highly significant (two tailed independent samples t-test, t=-11.570, d.f.=30, p<0.001). These decreases in pH of biofilms containing *S. mutans* are very important as the critical pH at which demineralization of teeth proceeds faster than remineralisation is 5.5 (when caries form, Dong et al. 1999), and because it appears the acidity of the environment kills the *V. dispar* (which is investigated later and the hypothesis is supported). Single species *V. dispar* biofilms have a pH of 7.56±0.14 after fourteen days (see Table 3.C), significantly different to the control biofilms which have a pH of 7.22±0.10 (two tailed independent samples t-test, t=4.564, d.f.=14, p<0.001).

Table 3.C. Summary statistics of total viable counts and pH for the different biofilms by day from the 10 *S. mutans* to 1 *V. dispar* biofilm experiment.

	Biofilm Growth (days)			
	1	3	7	14
TVC single-species *S. mutans*	$1.13x10^8 \pm 1.20 x10^8$	$4.65 x10^8 \pm 2.47 x10^8$	$1.81 x10^8 \pm 8.58 x10^7$	$3.48 x10^6 \pm 4.11 x10^6$
TVC dual-species *S. mutans*	$2.86 x10^8 \pm 1.91 x10^8$	$4.35 x10^8 \pm 1.55 x10^8$	$5.22 x10^7 \pm 5.04 x10^7$	$2.27 x10^6 \pm 3.26 x10^6$
TVC dual-species *V dispar*	$1.58 x10^8 \pm 9.91 x10^7$	$5.67 x10^7 \pm 5.56 x10^7$	$1.74 x10^6 \pm 1.78 x10^6$	$1.56 x10^2 \pm 6.25 x10^2$
TVC single-species *V dispar*	$7.32 x10^7 \pm 9.02 x10^7$	$6.10 x10^7 \pm 5.62 x10^7$	$5.29 x10^6 \pm 8.54 x10^6$	$5.94 x10^5 \pm 6.23 x10^5$
pH single-species *S. mutans*	6.42 ± 0.41	5.28 ± 0.22	4.87 ± 0.06	4.77 ± 0.04
pH dual-species biofilm	6.09 ± 0.55	5.41 ± 0.44	5.01 ± 0.11	5.02 ± 0.07
pH single-species *V. dispar*	7.37 ± 0.09	7.43 ± 0.04	7.48 ± 0.04	7.56 ± 0.14

Total viable counts and pHs are shown as mean ± one standard deviation. All total viable count mean and standard deviations have 16 replicates. All pH mean and standard deviations have 16 replicates except single-species *V. dispar* counts which have 14 replicates for one, three and seven day pH mean and standard deviations and 12 for the fourteen day pH mean and standard deviation.

3.3.3.1.3 Vitality of the bacteria in the 10 *S. mutans* to 1 *V. dispar* biofilm experiment

The percentage of live bacteria (vitality) was calculated for each time-point of each biofilm type from counts of fluorescently stained bacteria (see Figure 3.G). The vitality of both species declined over time. Single and dual species *S. mutans* showed similar patterns of decline although dual species *S. mutans* showed greater vitality throughout. Single species *V. dispar* showed a steady decline in vitality over the time-course, while dual species *V. dispar* displayed an early decrease in vitality before levelling off.

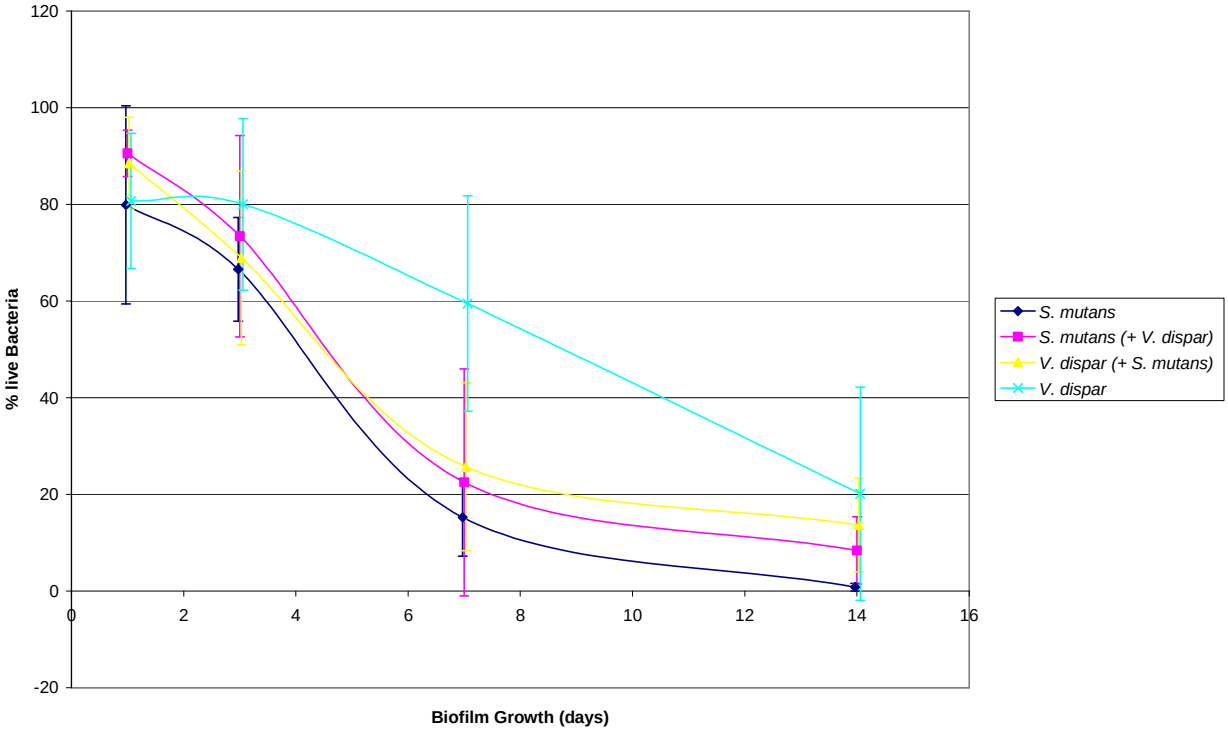


Figure 3.G. Vitality of the bacteria within the biofilms. Percent of each species of bacteria that were alive in the three types of biofilms for each of the four time points in the 10 *S. mutans* to 1 *V. dispar* biofilm experiment. The pink and yellow lines show the percentage of *S. mutans* and *V. dispar* bacteria (respectively) that were alive in the dual species biofilms. Error bars show one standard deviation.

The vitality of the biofilms was investigated using general linear models, using percentage of live bacteria of each species as the dependent factors and experiment (cohort), biofilm growth (days) and type of biofilm (*S. mutans* + *V. dispar*, and *S. mutans* or *V. dispar*) as the factors.

The first general linear model investigated the vitality of *S. mutans* and the model fitted the data very well (adjusted R^2 = 0.946) and was highly significant (F=72.463, d.f.=31, P<0.001). All factors and interactions of factors (except for biofilm growth*type of biofilm) were highly significant ([experiment F=28.907, d.f.=3, P<0.001], [biofilm growth F=658.442, d.f.=3, P<0.001], [type of biofilm F=28.861, d.f.=1, P<0.001], [experiment*day F=9.761, d.f.=9, P<0.001], [experiment*type of biofilm F=6.642, d.f.=3, P<0.001], [experiment*biofilm growth*type of biofilm F=5.186, d.f.=9, P<0.001]). This means that the experiment, the amount of biofilm growth, the type of biofilm and the way these factors (except for biofilm growth*type of biofilm) interacted were all important in determining the vitality of the biofilm and they could together explain 94.6% of the variation in vitality.

The second general linear model investigated the vitality of *V. dispar* and this model also fitted the data very well (adjusted R^2 = 0.940) and was highly significant (F=61.721, d.f.=30, P<0.001). All factors and interactions of factors were highly significant ([experiment F=20.910, d.f.=3, P<0.001], [biofilm growth F=395.800, d.f.=3, P<0.001], [type of biofilm F=61.899, d.f.=1, P<0.001], [experiment*day F=26.841, d.f.=9, P<0.001], [experiment*type of biofilm F=8.304, d.f.=3, P<0.001], [biofilm growth*type of biofilm F=28.895, d.f.=3, P<0.001], [experiment*biofilm growth*type of biofilm F=5.092, d.f.=8, P<0.001]). This means that the experiment, the amount of biofilm growth, the type of biofilm and the way these factors interacted were all important in determining the vitality of the biofilm and they could together explain 94.0% of the variation in vitality.

The vitality of the biofilms was assessed using fluorescence microscopy of bacteria stained with Live/Dead stain. There is a decline in the vitality of the bacteria in the biofilm and there is also much variation in the vitality of the bacteria for both species and all three biofilm types. The mean percentages of live bacteria in the biofilms were not significantly different for one day (oneway ANOVA, F=2.529, d.f.=3, P=0.066), or for three day biofilms (oneway ANOVA, F=1.793, d.f.=3, P=0.159). The mean percentages of live bacteria in the biofilm were significantly different for seven day biofilms (oneway ANOVA, F=16.093, d.f.=3, P<0.001) with single species *V. dispar* biofilms having significantly higher percentages of live bacteria in the biofilms than dual species *V. dispar* biofilms (P=0.001) and single (P<0.001) and

dual (P=0.001) species *S. mutans* biofilms. The mean percentages of live bacteria in the biofilm were significantly different for fourteen day biofilms (oneway ANOVA, F=7.167, d.f.=3, P<0.001) with single species *S. mutans* biofilms having significantly lower percentages of live bacteria in the biofilms than dual species *S. mutans* biofilms (P=0.004) and dual (P=0.001) species *V. dispar* biofilms. Single species *S. mutans* biofilms almost had a significantly lower percentage of live bacteria in the biofilm than single species *V. dispar* biofilms (P=0.057).

3.3.3.2 Effect of relative inoculum size on the interaction between *S. mutans* and *V. dispar* in biofilms

This experiment was a continuation of the previous experiment where 10 *S. mutans* to 1 *V. dispar* were used as the inocula. In this experiment 1/10 to 1, 1 to 1, and 1 to 1/10 ratios were investigated.

3.3.3.2.1 Biofilm relative inoculum size - total viable counts

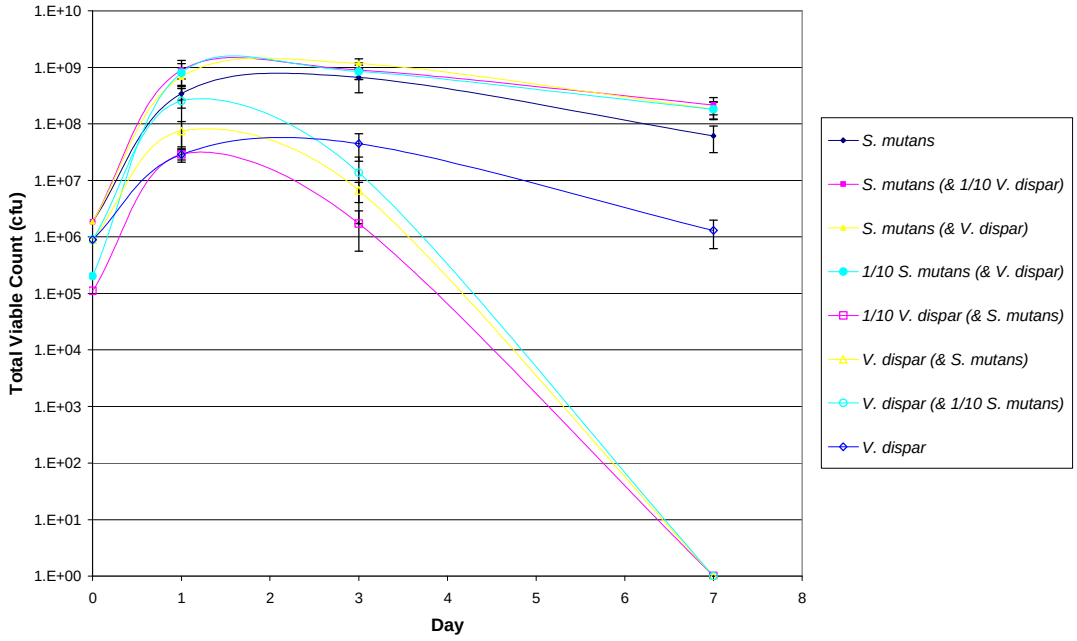


Figure 3.H. Total viable counts of both species of bacteria for each ratio from the relative inoculum size experiment. This figure shows plots of five different biofilm types, three of which had both species growing together. The three dual species biofilms are shown in pink, yellow and light blue, with *S. mutans* plotted with filled-in shapes and *V. dispar* plotted with outline shapes. In the dual species biofilms the species that is plotted is shown first in the legend and the species it grew with is in brackets. Differential innoculation ratios are shown by placing a fraction in front of the species name in the legend. Error bars show one standard deviation.

Figure 3.H shows how both species grew faster in the presence of the other than when grown alone up to the first day. By day 3 all *S. mutans* growing in the presence of *V. dispar* had higher population sizes than *S. mutans* growing alone, while the opposite relationship held for *V. dispar*. *V. dispar* growing in the presence of *S. mutans* had lower population sizes than *V. dispar* growing alone after 3 days. By day 7, all *V. dispar* that had been growing in the presence of *S. mutans* were dead, while *V. dispar* growing alone were surviving well. In contrast where *S. mutans* had been growing in the presence of *V. dispar* it still had higher population sizes than *S. mutans* growing alone after 7 days. When investigating the effect of inocula size, all dual species *S. mutans* curves were similar from day 1 irrespective of original inocula

ratios while *V. dispar* curves were all different. *V. dispar* that outnumbered *S. mutans* initially grew faster and reached higher population sizes than *V. dispar* that started off with the same inoculum as *S. mutans* which in turn reached a higher population size than *V. dispar* that started with a lower inoculum than *S. mutans*.

A general linear model was constructed to investigate total viable counts of *S. mutans*. This model initially contained all possible factors but each non-significant factor was removed until the model investigated the effect of biofilm growth and whether the *S. mutans* was growing as a single or dual species biofilm. *S. mutans* grew better as a dual species biofilm and this was highly significant ($F=36.372$, $d.f.=1$, $P<0.001$). Total viable counts varied depending upon length of biofilm growth and this was also highly significant ($F=97.611$, $d.f.=2$, $P<0.001$). This model was highly significant ($F=730.351$, $d.f.=11$, $P<0.001$) and these two factors (biofilm growth, single/dual species biofilm) explained 82.9% of the variation in *S. mutans* total viable counts (adjusted R^2). The starting inocula of *S. mutans* and *V. dispar* were not significant in determining the total viable counts of *S. mutans*.

The same process was conducted to investigate total viable counts of *V. dispar*. The model investigated the effect of biofilm growth, the starting ratio of the two species and the interaction of these two factors. The length of biofilm growth was highly significant ($F=3039.817$, $d.f.=2$, $P<0.001$) in determining the total viable counts of *V. dispar*. The starting ratios of *S. mutans* to *V. dispar* were also highly significant with single species biofilms having the highest counts and *V. dispar* outnumbered 10:1 by *S. mutans* initially having the lowest counts ($F=255.886$, $d.f.=3$, $P<0.001$) and the effect of these ratios changed over time as there was an highly significant interaction effect between starting ratio and length of biofilm growth with 1 day [10 *V. dispar*: 1 *S. mutans*] having higher counts than single species *V. dispar* and [1 *V. dispar*: 10 *S. mutans*] ($F=197.761$, $d.f.=6$, $P<0.001$). These changing dynamics can be seen in Figure 3.H. Dual-species *V. dispar* biofilms display higher r's and Ks initially but single species *V. dispar* biofilms survive better and have higher total viable counts from 3 days of growth as *V. dispar* in dual species biofilms collapse in numbers and are all dead by 7 days.

3.3.3.2.2 Biofilm relative inoculum size - pH

Unexpectedly, after three days, all dual species biofilms were more acidic than single species *S. mutans* biofilms, but by seven days, all biofilms containing *S. mutans* had stabilised at a pH of around 4.8 (see Figure 3.I). *S. mutans* can no longer grow at a pH of less than 4.8 (Quivey et al. 2001).

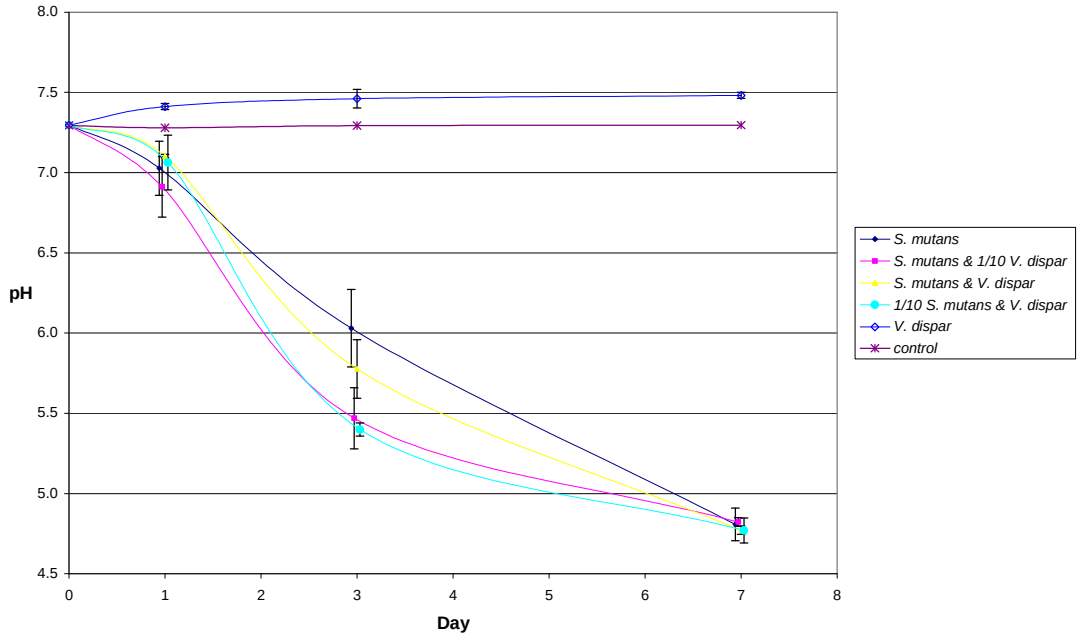


Figure 3.I. pH of the different biofilms from the relative inoculum size experiment.

This plot shows the pH of the five different biofilm types (three of which have the two species growing together) and of the control. All biofilm types containing *S. mutans* reach a similar pH, about 4.8, after seven days. Error bars show one standard deviation.

3.3.3.2.3 Biofilm relative inoculum size - summary

Table 3.D. Summary of growth of the biofilms in the relative inoculum size experiment.

	dN/dt	K (cfu)	σ of K (cfu)	Peak (days)
S. mutans	182	6.6×10^8	3.1×10^8	3
S. mutans & 1/10 *V. dispar*	478	8.9×10^8	2.9×10^8	3
S. mutans & *V. dispar*	364	1.2×10^9	2.4×10^8	3
1/10 ***S. mutans*** & *V. dispar*	3931	8.4×10^8	2.3×10^8	3
S. mutans & 1/10 ***V. dispar***	260	2.9×10^7	6.0×10^6	1
S. mutans & ***V. dispar***	83	7.4×10^7	3.5×10^7	1
1/10 *S. mutans* & ***V. dispar***	285	2.5×10^8	2.2×10^8	1
V. dispar	31	2.9×10^7	7.7×10^6	1

Values are shown for the species shown in bold in the left column which also indicates whether the species was growing alone or with the other species at a set ratio. Rates of growth dN/dt, maximum population sizes (carrying capacities, K) and σ (standard deviation) of K are shown.

dN/dt does not show exponential growth, as the period also includes limited growth. When *S. mutans* is grown in dual species biofilms with a starting inoculum 1/10 of the other dual species biofilms, dN/dt is approximately 10 times higher. *V. dispar* grew well when it outnumbered *S. mutans* initially as the rate *V. dispar* removed lactic acid kept up with the rate of production by *S. mutans* for a little longer.

3.3.3.3 Effect of D-glucose concentration on the interaction between *S. mutans* and *V. dispar* in biofilms

Biofilms were grown on media with five different D-glucose concentrations (0g/L, 0.025g/L, 0.25g/L, 2.5g/L and 25g/L). This next subsection details the result of this experiment. The glucose concentrations used are biologically relevant as there is "practically no glucose" present in the saliva normally (Meurman et al. 1987) and levels between 1g/L and 126g/L 60 minutes after eating a chocolate bar and between 11g/L and 144g/L 60 minutes after eating white bread (Luke et al. 1999).

3.3.3.3.1 Effect of D-glucose concentration on the total viable counts of *S. mutans* and *V. dispar* in biofilms

Total viable counts taken for the different biofilms growing with different treatment concentrations of D-glucose are shown in Figure 3.J.

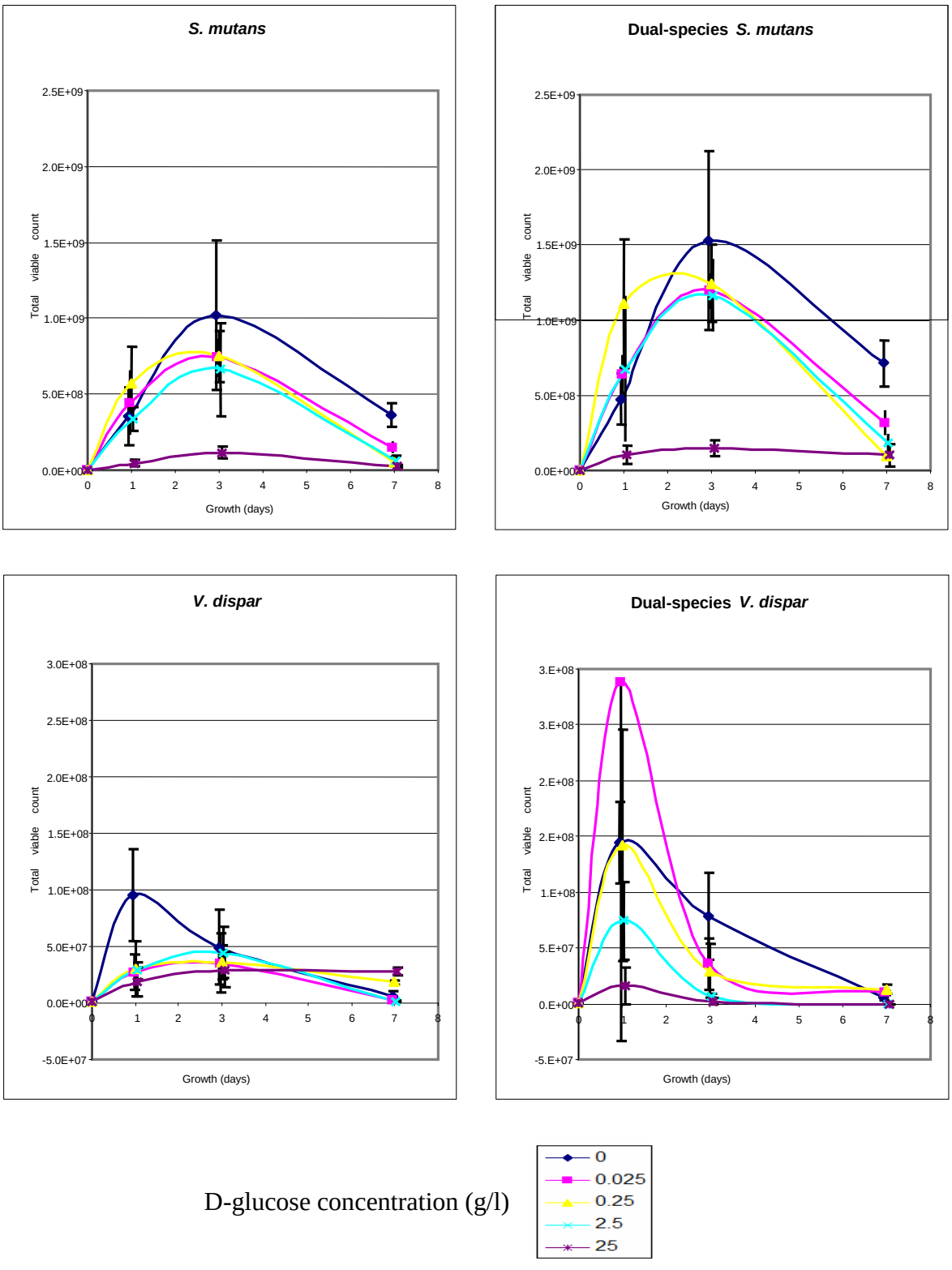


Figure 3.J Total viable counts of single and dual species biofilms with different D-glucose concentrations in the media. Error bars show one standard deviation.

S. mutans displays similar curves, but these are shifted up (the counts are higher) in dual-species biofilms. *V. dispar* displayed the highest K in dual species biofilms at a D-glucose concentration of 0.025g/L and lower Ks the further from this concentra-

tion the treatments were. Single-species *V. dispar* biofilms were very similar except the treatment with no D-glucose had a higher dN/dt and K.

A general linear model was constructed to analyse total viable counts of *S. mutans.* The model initially contained all possible factors and each non-significant factor was removed until the model investigated the effect of D-glucose concentration, biofilm growth, whether *S. mutans* was growing as a single or dual species biofilm, the effect of the covariate pH and the interaction of the factors D-glucose concentration and length of biofilm growth. D-glucose concentration was highly significant ($F=4.527$, d.f.=4, $P<0.001$). The length of biofilm growth was highly significant ($F=110.236$, d.f.=2, $P<0.001$). Whether *S. mutans* was growing in single or dual species biofilms was highly significant with dual species biofilms having higher total viable counts ($F=75.970$, d.f.=1, $P<0.001$). The covariate pH was significant ($F=4.462$, d.f.=1, $P=0.037$) and the interaction of D-glucose concentration and biofilm growth was highly significant ($F=14.716$, d.f.=8, $P<0.001$). The model itself was highly significant ($F=57.516$, d.f.=16, $p<0.001$) and explained 88.4% of the variation in *S. mutans* total viable counts (adjusted R^2).

A general linear model was constructed to analyse total viable counts of *V. dispar.* The model initially contained all possible factors and each non-significant factor was removed until the model investigated the effect of D-glucose concentration, biofilm growth, the effect of the covariate pH and all possible interactions of the factors D-glucose concentration, length of biofilm growth and whether *V. dispar* was growing as a single or dual species biofilm. Note that whether *V. dispar* was growing as a single or dual species biofilm was only included in interaction effects with other factors and not as a factor on its own. D-glucose concentration was highly significant ($F=29.878$, d.f.=4, $P<0.001$). The length of biofilm growth was highly significant ($F=110.892$, d.f.=2, $P<0.001$). The covariate pH was highly significant ($F=13.051$, d.f.=1, $P=0.001$). The interaction of D-glucose concentration and biofilm growth was highly significant, ($F=33.128$, d.f.=8, $P<0.001$). The interaction of D-glucose concentration and whether *V. dispar* was growing as a single or dual species biofilm was highly significant (dual species biofilms with D-glucose concentrations of 0.025 and 0.25 g/L higher total viable counts, $F=21.917$, d.f.=4, $P<0.001$). The interaction of the length of biofilm growth and whether *V. dispar* was growing as a single or dual species biofilm was highly significant (one and three day dual species counts higher,

F=49.013, d.f.=2, P<0.001). And finally the interaction of D-glucose concentration, biofilm growth and single/dual species was highly significant ([one day dual species biofilms with D-glucose concentrations of 0.025g/L and 0.25g/L, 3 day dual species biofilms with D-glucose concentrations of 2.5g/L and seven day dual species biofilms with D-glucose concentrations of 0.025g/L all had higher counts than their single species equivalent and seven day single species biofilms with a D-glucose concentration of 25g/L had higher counts than their dual species equivalent], F=33.443, d.f.=8, P<0.001). The model itself was highly significant (F=2184.966, d.f.=31, p<0.001) and explained 99.8% of the variation in *S. mutans* total viable counts (adjusted R^2).

Both *S. mutans* and *V. dispar* have higher total viable counts with a D-glucose concentration of 0g/L in dual species biofilms after three days than they do as single species biofilms although not significantly so. Although not significant, this is interesting for two reasons. Firstly, the availability of D-glucose reduces the survival of both species in a closed system, and secondly even without D-glucose, both species are still benefiting from the presence of the other species despite the lack of a shared metabolic pathway indicating there are other benefits to co-culture unrelated to the production and removal of lactic acid.

3.3.3.3.2 Effect of D-glucose concentration on the pH of *S. mutans* and *V. dispar* biofilms

pH showed the same trends as in the 10 *S. mutans* to 1 *V. dispar* experiment (see Figure 3.K) except the medium with 25g/L was slightly acidic (pH=6.6). Because of the large amount of D-glucose it was autoclaved at 115ºC rather than being filter sterilised and the D-glucose reacted to form slightly acidic products. It is unknown why this occurred but media containing 25g/L glucose had a pH of about 6.6 rather than 7.2. Also *S. mutans* biofilms growing with D-glucose concentrations of 0.25g/L or below did not stray that far from neutral pH (all > pH 6.7). It was in dual species biofilms at these D-glucose concentrations that *V. dispar* thrived.

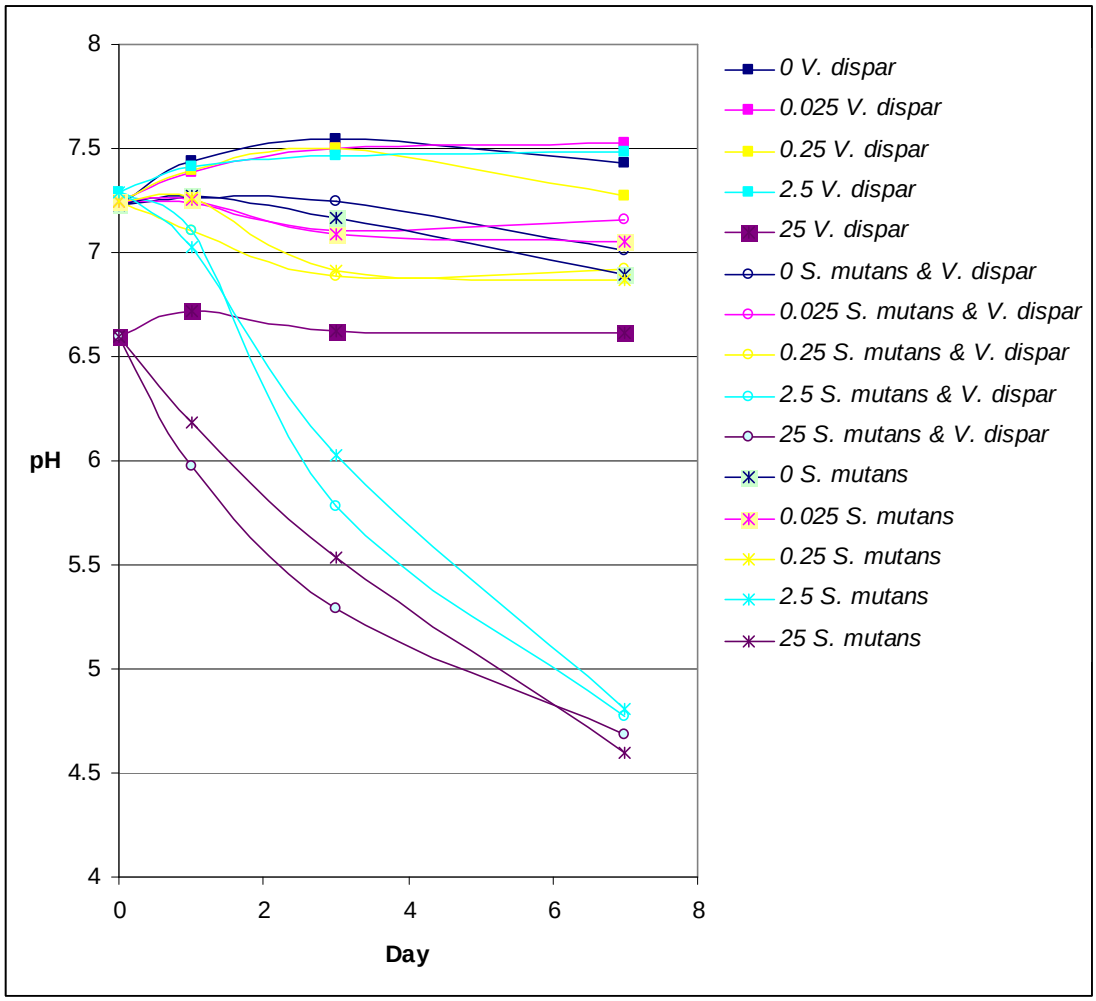


Figure 3.K. pH of the biofilms in different concentrations of glucose.

The mean pH of fifteen different types of biofilms are plotted for zero, one three and seven days of growth. The three biofilm types (the two species growing separately and together) are plotted for five different glucose concentrations. Each of the three biofilm types is connected by lines of the same colour for each glucose concentration (e.g. dark blue lines for the three biofilm types with no glucose). *V. dispar* biofilms are plotted with solid boxes, dual species biofilms with circles and *S. mutans* biofilms with crosses.

Table 3.E. Summary of growth of the different biofilms with different D-glucose concentrations

Biofilm	D-glucose	Final pH	σ of Final pH	dN/dt	K (cfu)	σ of K (cfu)	Peak (days)
S. mutans	0	6.89	0.02	190	1.0×10^9	4.9×10^8	3
	0.025	7.05	0.02	238	7.4×10^8	1.3×10^8	3
	0.25	6.87	0.07	308	7.5×10^8	1.7×10^8	3
	2.5	4.81	0.10	182	6.6×10^8	3.1×10^8	3
	25	4.60	0.08	23	1.2×10^8	4.2×10^7	3
S. mutans & *V. dispar*	0	7.01	0.02	256	1.5×10^9	5.9×10^8	3
	0.025	7.15	0.01	343	1.2×10^9	1.2×10^8	3
	0.25	6.92	0.02	597	1.2×10^9	2.6×10^8	3
	2.5	4.77	0.03	364	1.2×10^9	2.4×10^8	3
	25	4.68	0.07	56	1.5×10^8	5.0×10^7	3
S. mutans & ***V. dispar***	0	7.01	0.02	162	1.4×10^8	3.7×10^7	1
	0.025	7.15	0.01	324	2.9×10^8	3.2×10^8	1
	0.25	6.92	0.02	160	1.4×10^8	1.0×10^8	1
	2.5	4.77	0.03	83	7.4×10^7	3.5×10^7	1
	25	4.68	0.07	17	1.6×10^7	1.7×10^7	1
V. dispar	0	7.43	0.01	106	9.5×10^7	4.1×10^7	1
	0.025	7.53	0.02	30	3.5×10^7	2.6×10^7	3
	0.25	7.27	0.12	33	3.6×10^7	1.5×10^7	3
	2.5	7.48	0.02	31	4.4×10^7	2.3×10^7	3
	25	6.62	0.10	20	3.0×10^7	1.5×10^7	3

Values are shown for the species shown in bold in the left column which also indicates whether the species was growing alone or as a dual species biofilm. pH, rates of growth (dN/dt), maximum population sizes (carrying capacities, K), day when K reached (Peak) and σ (standard deviation) of pH and K are shown for each of the five D-glucose concentrations used.

3.3.3.4 Interaction between *S. mutans* and *V. dispar* on a buffered medium

In this experiment biofilms were grown on buffered and unbuffered medium to investigate the effects of buffering pH on the interactions of these two species. This experiment was attempted twice (using different concentrations and types of buffer: MES, MOPS, phosphate buffer) and there were problems with the growth of *V. dispar* on the buffered medium (data not shown). There was an issue with agar

setting due to the high concentrations of buffer that were needed to cope with the amount of lactic acid that was produced, and this resulted in *V. dispar* not growing.

3.3.4 Effect of Lactic Acid on Growth of *V. dispar*

Lactic acid concentration and buffering pH were found to be highly significant in their effect on *V. dispar* growth (oneway ANOVA, $F=13.133$, d.f.=9, $P<0.001$). Lactic acid increases the growth rate and bacterial yield of *V. dispar* with the maximum growth detected in this study occurring at a concentration of 140mMol lactic acid in a solution buffered to a pH of 7.2 (P values ranged from 0.027 to 0.316 for the comparisons of whether 140mMol was larger for the nine comparisons against other means for the final time point, equivalent concentration to the "21 ml/litre of 60% lactic acid syrup" used by Palmer et al. 2006). Lactic acid at a concentration of 50mMol inhibits *V. dispar* growth in unbuffered medium (P values ranged from <0.001 to 0.129 in the nine comparisons of whether 50mMol was lower, see Figure 3.L, particularly yellow triangles and maroon circles showing the effect of buffering lactic acid at a concentration of 50 mMol). These data support the conclusion that the lactic acid produced by *S. mutans* in dual species biofilms accumulates to a concentration that kills off the *V. dispar* at the later time points.

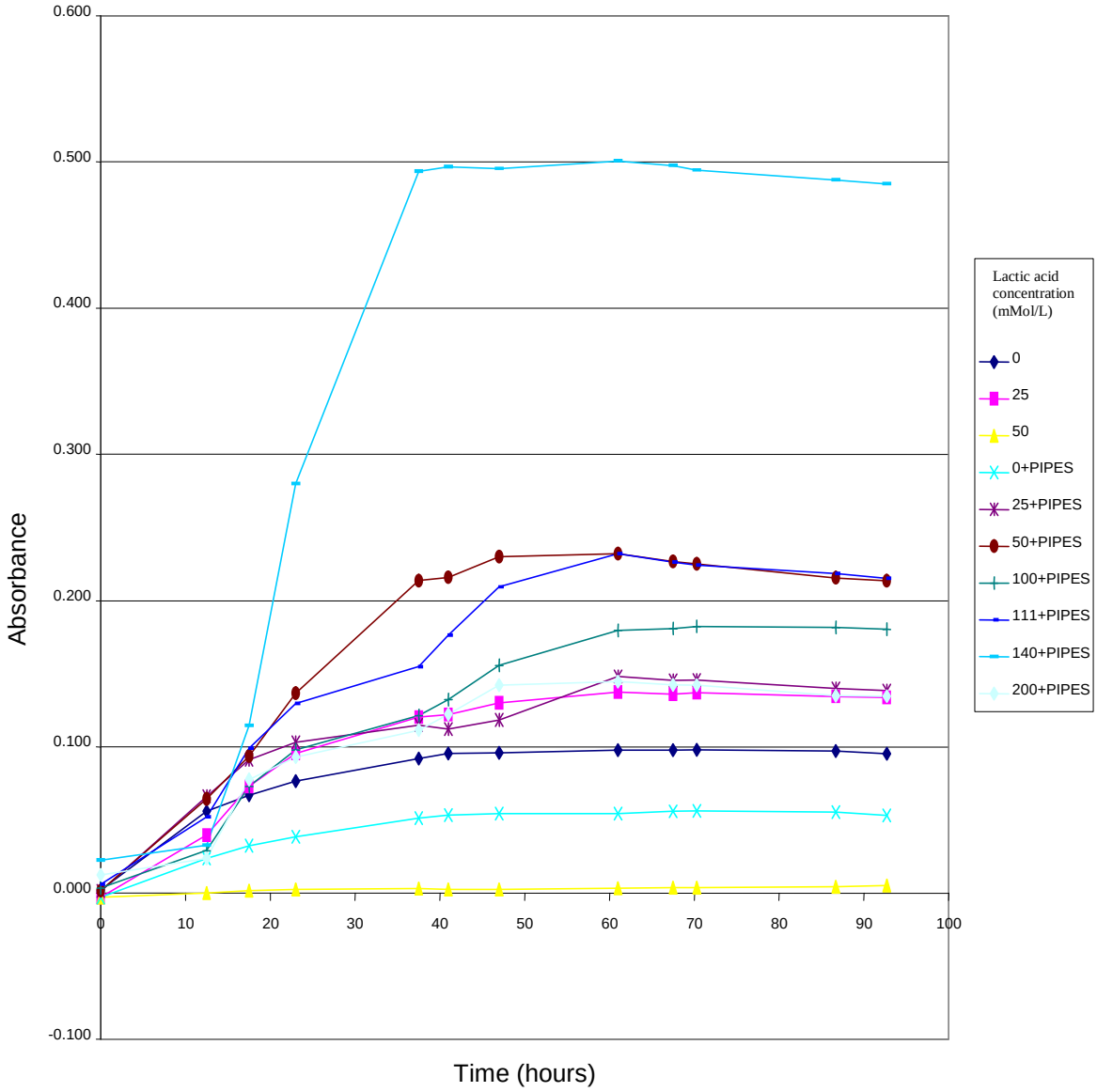


Figure 3.L. The effects of lactic acid and buffering pH on the growth of *V. dispar*. *V. dispar* growth curve for different lactic acid concentrations (mMol/L) in buffered (PIPES, 50mMol/L) and unbuffered media. Each data point is the average of six samples. Absorbance readings were taken at 590nm.

3.3.5 Summary of Results

This study investigated how *S. mutans* and *V. dispar* interacted with each other and their environment.

- *S. mutans* lowered the pH of the environment through the metabolism of glucose to lactic acid.
- *V. dispar* raised the pH of the environment through the metabolism of lactic acid to weaker acids and through the breakdown of nitrate and nitrogenous bases to alkaline products.
- The decrease in pH of the environment caused by *S. mutans* limited the growth of *S. mutans* and killed *V. dispar*.
- The rise in pH caused by *V. dispar* was small but in certain circumstances it was sufficient to be beneficial; in particular when *V. dispar* outnumbered *S. mutans* and when there was limited glucose in the system which limited the yield of lactic acid that could be produced by *S. mutans*.
- In general the presence of *V. dispar* had little effect on *S. mutans*, and this only occurred when *V. dispar* could make a significant difference to the pH of the environment in the circumstances outlined above.
- *V. dispar* was killed by high concentrations of lactic acid and the resulting low pH, however *V. dispar* benefited from the presence of low concentrations of lactic acid, and thus in some of the systems investigated *V. dispar* benefited from the presence of *S. mutans*.
- *V. dispar* benefited from the presence of *S. mutans* in buffered and unbuffered planktonic growth.
- When growing as a biofilm, *V. dispar* did not benefit from the presence of *S. mutans* if the time-course was investigated as a whole but if the relationship was broken down to the different stages of biofilm growth, *V. dispar* benefited initially before lactic acid accumulated, and suffered once lactic acid had accumulated.
- When glucose concentrations were varied, *V. dispar* could benefit from the presence of *S. mutans* if there was a sufficiently limited glucose supply such that it could not be metabolised to a dangerous concentration of lactic acid.
- *S. mutans* showed a slight but non-significant increase in vitality in the presence of *V. dispar* in both planktonic and biofilm growth.

- *V. dispar* showed a non-significant decrease in vitality in the presence of *S. mutans*.
- *S. mutans* appeared to show a growth rate versus growth yield trade-off as when it was grown without D-glucose it achieved a higher growth-yield but it used D-glucose when it was present to achieve a higher growth-rate.

3.4 Discussion

3.4.1 Purpose of Study

Bacteria exist in multi-species communities so this study investigates the interactions that occur between two key species in an important bacterial community, dental plaque. As bacteria rarely exist as a monoculture it is important to understand how they interact with each other and this study investigated co-culture of *S. mutans* and *V. dispar* in liquid medium and as biofilms. The primary aim of this study was to determine how these two species interact and what drives these interactions. This knowledge is important to understand these bacteria and bacteria in general and it may identify ways to beneficially modulate bacterial communities. It is becoming increasingly apparent that some disease states are caused by disruptions in the composition of bacterial communities, with changes in the ratios of different species rather than the presence of individual species. Often pathogenic bacteria are present but do not cause disease, as is the case with *S. mutans* in the absence of sugars. Once the factors that cause benign communities of bacteria to develop in to pathogenic communities are identified, these factors can be directly targeted, in addition to or instead of the pathogens themselves. An abundant supply of sugar is one such factor and it and other environmental factors that allow pathogenic bacteria to gain dominance can be considered as 'ecological catastrophes' (Marsh 2003).

This study investigated growth of these species as planktonic cultures in liquid medium and as biofilms growing at a gas/solid nutrient interface. Many studies investigate biofilm or planktonic growth in 96 well plates. The problem with this is immediately apparent, as both types of growth may occur in 96 well plates. Thus, to investigate the interactions that occur in planktonic or biofilm growth two different methods were used. Planktonic growth was investigated in 50 ml falcon tubes, where the volume to surface area (50mL volume to 108.3cm^2 surface area [3.0cm x π x 11.5cm], 0.46ml/cm^2) is much greater than in a 96 well plate (200uL volume and 2.7cm^2 [Thermo Scientific data-sheet] surface area, 0.07ml/cm^2), thus minimising the effect of surface growing biofilms on the results. A method for growing biofilms was developed where there is no possibility of planktonic growth, by growing the bacteria at a gas/solid medium interface, and methods for assaying bacteria growing in this way were developed. This biofilm growth model differed from the conven-

tional methods of biofilm growth (constant depth film fermenter [CDFF], flow cell, n-well plate) as it allows no planktonic growth. It also differs from CDFF and flow cells in that it is a semi-closed, static system where nutrients get depleted and wastes accumulate. As it is a semi-closed system, nutrients provided can be tightly regulated and modified easily. Furthermore, the waste products produced remain in the system where they can affect the interactions and growth of the species.

3.4.2 General Discussion of Findings

These experiments partially support the hypothesis that these two species benefit from the presence of each other (have a mutualistic relationship) because of their respective roles in the production and removal of lactic acid, but only under certain conditions. Lactic acid is known to kill *V. dispar* and limit the growth of *S. mutans*, and this study has shown this could underlie a possible growth yield trade-off for *S. mutans* in the metabolism of D-glucose. This study highlights that there are limited circumstances within which these two species both benefit, and that the dynamics of the interactions that occur change over time. In liquid medium, where all nutrients and waste diffuse rapidly, *S. mutans* gained no benefit from the presence of *V. dispar*, but *V. dispar* did benefit from the presence of *S. mutans*. However in the highly structured environment of a biofilm, *S. mutans* could benefit from the presence of *V. dispar* if the conditions were such that *V. dispar* was not rapidly killed by low pH. *V. dispar* benefited from, and was eventually killed by, the presence of lactic acid in the medium. Thus *V. dispar* showed changing dynamics of interactions with *S. mutans*, encompassing mutually beneficial, commensal, neutral and competitive depending upon its spatiotemporal environment (see Table 3.F).

Table 3.F. Summary of interactions with regard to total viable counts.
This table summarises whether each species benefitted from the presence of the other species when they were co-cultured for each of five different experiments. The different experiments are shown in columns and the effect on each species is shown in rows. The interactions were classed as

- is negative, **n.e.** is no effect, and **+** is positive

Species	Planktonic		Biofilm		
	unbuffered	buffered	10 *S. mutans* to 1 *V. dispar*	ratio	D-glucose
S. mutans	**n.e.** 3 day -	**n.e.** 1 day Acetate +	**n.e.** 7 day -	**+**	**+**
V. dispar	**+**	**+**	1 day + 14 day -	**-** 1 day S:10V +	**+** 7 day 25g/L -

Table 3.F shows that *V. dispar* benefitted from the presence of *S. mutans* (had higher total viable counts when grown with *S. mutans* than when it was growing alone and thus + shown in the table) in both unbuffered and buffered planktonic growth experiments. However when growing as a dual species biofilm with *S. mutans*, *V. dispar* had negative interactions (ratio experiment), positive interactions (D-glucose experiment) and negative and positive interactions (10 *S. mutans* to 1 *V. dispar* experiment showed *V. dispar* had higher total viable counts when grown as a dual species biofilm after 1 day but lower after 14 days when compared with total viable counts of *V. dispar* growing as a single species biofilm).

This study has identified how the dynamics of species interactions change in these planktonic and biofilm communities. The results highlight the variation that exists between the different samples and treatments. However, some clear patterns emerged. Typically, in the biofilm experiments, after one day of growth, both *S. mutans* and *V. dispa*r had higher total viable counts when grown with the other species than when they were grown alone. Both species were benefiting from the presence of the other. After three days of growth, there were no differences in total viable counts between the species growing separately and together. After seven days

of growth *V. dispar* had a lower total viable count when it was grown with *S. mutans* than when it was grown alone and after fourteen days there were almost no *V. dispar* left in dual species biofilms.

Acidity built up rapidly in all unbuffered broths and biofilms containing *S. mutans*. This decrease in pH causes the decrease in *V. dispar* total viable counts in dual species treatments as demonstrated by the experiment investigating the growth of *V. dispar* with lactic acid in buffered and unbuffered media. The 10 *S. mutans* to 1 *V. dispar* biofilm experiment demonstrated that *V. dispar* could not metabolise lactic acid as fast as it was being produced, and the acid that built up killed it. This supports the work of Distler and Kröncke (1980) who highlighted the importance of metabolic velocity in the interaction of *S. mutans* and *Veillonella*. They showed *S. mutans* rapidly produced lactic acid but that *Veillonella alcalescens* could only process it slowly and consequently lactic acid could accumulate, as occurred in these experiments. In the next two biofilm experiments, the amount of D-glucose available and the starting ratios of *V. dispar* to *S. mutans* were investigated as to whether there were favourable conditions to prolong the mutually beneficial relationship. Both limiting the D-glucose in the system and thus the lactic acid, and increasing the relative numbers of *V. dispar* metabolising the lactic acid did indeed prolong the mutual benefit. When *V. dispar* can not process the lactic acid as fast as *S. mutans* produces it the system becomes more acidic. The experimental studies showed that *S. mutans* rapidly lowered the pH and when this occured the symbiotic relationship ceased.

The biofilm growth model in this system was a semi-closed system (only gases exchanged with the external environment), where nutrients were depleted and waste products accumulated. This growth model was used so that lactic acid had the potential to accumulate. When lactic acid did accumulate this caused *Veillonella* numbers to drop which is not the typical interaction reported in the literature (Bradshaw and Marsh 1998, Egland et al. 2004, Kara et al. 2006). Many models of biofilm formation investigate open systems where nutrients are replenished and waste products are removed or alternatively buffer the system. The dental plaque biofilm is an open system with biofilms bathed in saliva. This saliva would wash away some of the accumulated lactic acid, which would also diffuse through the saliva (Hicks et al. 2003). In addition to saliva flushing the system, in the natural environment there are

other bacteria competing with *S. mutans*, and more metabolites present that would have a buffering effect (Hicks et al. 2003). These effects may combine to reduce the likelihood of the dangerous drop in pH and the steep decline in *V. dispar* numbers that were observed in many of the experiments reported in this chapter. In a similar study to this, where dual species biofilms containing *S. mutans* and *Veillonella parvula* were grown in buffered medium, lactic acid concentrations were reduced in the dual species biofilms (Kara et al. 2006). A mixed culture chemostat study including *S. mutans* and *Veillonella* species showed *Veillonella* species were always the most numerous after D-glucose pulsing (Bradshaw and Marsh 1998). A different *Veillonella* species, *Veillonella atypica*, upregulated lactic acid production in a different *Streptococcus* species thus indicating lactic acid was limited rather than limiting (Egland et al. 2004). This implies that *V. atypica* was metabolising all the lactic acid available.

S. mutans can metabolize a wider variety of carbohydrates than any other Gram-positive bacteria sequenced to date (Ajdić et al. 2002). This study used a complex rather than a defined medium which would be closer to the conditions found in the dental plaque biofilm as complex medium contains many different nutrients. Metabolising D-glucose will eventually limit growth if enough D-glucose is present because of the accumulation of lactic acid. However healthy subjects display a range of 0-225mg/L (0-1.25mMol/L) glucose in saliva and similar levels for gingival crevicular fluid (GCF, Meurman et al. 1987, Luke et al. 1999, Ciantar et al. 2002, Di Gioia et al. 2004) which is similar to the range of glucose concentrations where the interactions of these two species are beneficial.

When D-glucose was absent, *S. mutans* grew well. It appears *S. mutans* displays a growth yield trade-off with regard to D-glucose use that always favours growth if possible. This makes evolutionary sense as if any *S. mutans* bacteria employ the growth strategy, of metabolising D-glucose, this produces lactic acid which eventually becomes toxic and limits the yield of all *S. mutans* whether they have employed this strategy or not. Any *S. mutans* using other metabolic pathways utilising different nutrients will have grown slower, and will be fewer in number once growth is limited. This is an example of the 'tragedy of the commons' (Hardin 1968, MacLean 2008), but rather than the typical case where individuals acting in their own self-interest overexploit a communal resource, individuals are polluting a communal

environment. Hardin (1968) refers to pollution as a reverse example of the tragedy of the commons referencing human examples of sewage, radioactive waste and noxious fumes while Rankin et al. (2007) claim that pollution is the overexploitation of the environment and is thus the normal case of the overexploitation of a communal resource. The metabolism of D-glucose is famous as an example of a growth yield trade-off in yeast, where D-glucose can be metabolised anaerobically for low yield but at a high rate, or metabolised aerobically (which *S. mutans* can not do) for a high yield but at a low rate. Kin selection where behaviours are displayed if they benefit close relatives can prevent the tragedy if environments are structured (MacLean 2008). Biofilms are highly structured and it has been demonstrated in these highly structured environments that biofilms enrich altruists and that growth yield is preferable (Kreft 2004). However the highly structured biofilms in these studies still displayed a growth rate, rather than yield, preference presumably because lactic acid diffuses so readily through this structured environment. It is interesting that in these populations where *S. mutans* was almost 100% related to all the other *S. mutans* growing, kin-selection did not select it to employ a strategy favouring maximum population and survival over growth. Maximising growth rate won. An alternative view is that lactic acid is not a waste product at all but rather a chemical warfare agent and this hypothesis is presented in Section 5.4.2.

Many species normally coexist. Each has their own niche. The presence of other bacteria is not inhibitory unless they compete for a limited resource or poison each other by their waste products. West et al. (2006) state that the use of the waste product of one individual by another is the simplest scenario that can lead to the evolution of cooperation, as a selfish behaviour benefits another and West et al. call this a one-way by-product benefit. This is the case with the system described in this report. This study demonstrates a symbiotic relationship that breaks down because of the increase in acidity. The irony of this is that the lactic acid that fuels the symbiotic relationship is the cause of the negative relationship that ensues. Some *Veillonella* species can survive at a pH of 4.5 (Wilson 2005) and these may become more predominant in the dental plaque biofilm as acidity increases. This would be a typical pattern of succession.

S. mutans grew fastest in neutral buffer, so even though it is acidophilic, it still, like the majority of bacteria, grows fastest at neutral pH. The intracellular pH of *S.*

mutans is actually 7.5 despite it lowering the pH of the environment through the metabolism of its primary energy source, D-glucose (Quivey et al. 2000, 2001). *S. mutans* has a minimum pH for growth of 4.8 yet its minimum pH for glycolysis is 4.4 (Bender et al. 1985). pH in the unbuffered biofilms and broths containing *S. mutans* and sufficient D-glucose stabilised at 4.8 in these experiments indicating the bacteria stopped metabolising D-glucose to lactic acid when they stopped growing. In contrast, even though *V. dispar* is killed by a pH of 5.1 at which *S. mutans* can grow, *V. dispar* grew better at a pH of 6.1 than at neutral pH. *S. mutans* displays a wide range of pH tolerance and is known to possess two different mechanisms to cope with decreases in pH of the environment (Quivey et al. 2001). Reducing the pH of the environment is actually adaptive for *S. mutans* as it will exclude other less acidophilic bacteria including closely related competitors *S. gordonii* and *S. oralis* although this requires the pH to decrease below pH 4.5 which did not occur in this study (Bradshaw and Marsh 1998). *S. mutans* initially decreased in numbers in an alkaline environment (Tricine buffer, pH 8.1) but then recovered and grew. During this decline it would have been adapting to the environment and was probably producing alkaline-shock protein (SMU.228) to allow it to survive (Ajdić et al. 2002).

The analysis of vitality within the different biofilms showed there is a lot of variation within the same biofilm type. There was a general decrease in the vitality over time as expected, but no clear difference between single and dual species biofilms but, interestingly, a much faster decrease in vitality within the biofilms compared with the decrease in planktonic cultures. This could be as a result of kin selection favouring apoptosis for the good of relatives or more simply due to local patches of starvation or toxic poisoning due to the high cell density. At the community level biofilms were more resilient than planktonic cultures as the pH changes that occurred (*V. dispar* making the environment more basic and *S. mutans* making the environment more acidic) as a consequence of the biofilm phenotype were more extreme (the pH changed more) than the equivalent planktonic phenotype experiment.

As this study investigated the interaction of these two species it raises the question; are they influencing each other directly or are they changing the environment which in turn influences the other species? The major effects are certainly changes to the environment (indirect effects); the addition of a food source and consequent reduc-

tion of pH. However in the biofilm experiments *S. mutans* benefited while it did not in the liquid culture experiments suggesting a spatial or structural component of the interaction. Either *S. mutans* is benefiting from assistance in producing structural products (e.g. excreted DNA) for the biofilm or some similar effect or it is benefiting from localised spatial effects caused by the biofilm. It is possible these effects are direct. A different *Veillonella* species is known to directly signal a different *Streptococcus* species (Egland et al. 2004). The gene expression study detailed in Chapter 5 investigated whether there were any genetically controlled responses (behaviours) employed by *S. mutans* in response to the presence of *V. dispar* and its metabolites.

It is interesting to see the changing dynamics of this system as the biofilms grow and mature, particularly how a symbiotic relationship can become 'acidic'. It is also interesting to note that the two species did not appear to be competing for any nutrients or for space. Both must have been limited by different things. After three days of growth it appears the species were neither cooperating nor competing. It would be interesting to repeat a similar experiment with *S. mutans* and a competitor (such as a different type of streptococci that is found in the oral cavity) to compare how they grow together and alone.

This study only investigated single and dual species biofilms. However the dental plaque biofilm is estimated to comprise as many as 20,000 phylotypes (Keijser et al. 2008). Almost all of these are uncultured, probably because of the very specific niches they have, and the relationships occurring in this system are probably required for the cultivation of some of these. While there is unlikely to be this many phylotypes at any one time, there will be many species of bacteria in typical plaque. As the dental plaque biofilm increases in complexity, space will become increasingly limited. This would mean growth of individual species would be constrained by space as well as by nutrients. *S. mutans* might not be able to increase to a population where it can produce enough lactic acid to cause a collapse. This could mean that the system itself would 'buffer' against extreme perturbations, such as is seen with the increase in acidity in many of the dual-species systems investigated. The food web produced would be incredibly complex, and the system might become more robust. Communities of many different species living in a constant state of conflict and sometimes cooperation is the norm in nature, be the species bacteria or animals and plants.

4 Biofilm Microscopy Studies

4.1 Introduction

Microscopic techniques are useful for determining structural and architectural parameters and identifying structure-function relationships (Wolf et al. 2002). This chapter describes the microscopic investigations conducted to determine if there are any interesting spatial interactions occuring or differences in patterns of growth in space between single and dual species biofilms. It was also conducted to determine how spacial interactions should be modelled (if at all). Confocal and 2-Photon microscopy use lasers and fluorescent molecules to image biological samples in three dimensions and this can be done with living samples. Scanning Electron microscopy uses electrons rather than photons (light) to image samples. Electrons have a smaller amplitude and wavelength than photons and hence provide better resolution and possible magnification than those achievable by light microscopy. However, samples need to be coated in a thin layer of metal which means that living samples can not be observed. Confocal microscopy is ideal for investigating living biological samples as it is a relatively simple procedure and, as the samples can be visualised in three dimensions without the necessity for dehydration, it enables the true structure to be visualised.

This chapter describes the biofilm architecture, vitality, and distribution of live and dead bacteria of dual-species *S. mutans* and *V. dispar* biofilms and single-species biofilms of each species that were grown on membrane filters placed on agar. It also attempts to infer how the biofilms grow and how their architecture changes over time as biofilms were scanned using confocal microscopy at one, three, seven and fourteen days. Biofilms were also scanned after one day of growth using scanning electron microscopy.

4.2 Materials and Methods

Biofilm structure studies were conducted as outlined in the Materials and Methods chapter. The structure and vitality of single and dual species biofilms were investigated as outlined in section 2.4.1 (using confocal microscopy). The morphology of single and dual species biofilms was investigated as outlined in section 2.4.2 (using scanning electron microscopy).

Z-projects were created for all image stacks using ImageJ, for both the green (live bacteria) and red (dead bacteria) channels. These were collapsed from three into two dimensions, preserving the maximum intensity identified in the z-dimension in the new x-y images. The two images were then merged into a single RGB image but with the red channel converted to blue (for colour blind readers). Quantification of fluorescence intensity was done using ImageJ (Abramoff et al. 2004).

4.3 Results

4.3.1 Confocal Microscopy

4.3.1.1 Structure and vitality of the biofilms in the 10 *S. mutans* to 1 *V. dispar* biofilm experiment (when *S. mutans* initially outnumbered *V. dispar* 10 to 1)

Structure and vitality of the biofilms were investigated using confocal microscopy of the biofilms at each time point. Figure 4.A shows an x-y slice of a one day old dual-species *S. mutans* and *V. dispar* biofilm with x-z and y-z slices shown at the sides, and using the method outlined in Figure 4.B, Figure 4.C is a montage of all the x-y slices (the z-series; sampled every 2µm of depth) of the same biofilm. The normal Live(green)/Dead(red) stain has been represented in different channels Live(green)/Dead(blue) due to the high prevalence of green/red colour-blindness. These figures show the biofilm forms a lawn of approximately 20µm thickness (10 slices in the montage where the same pixel fluoresces multiplied by the 2µm step between slices). Three dimensional structures are difficult to represent in two dimensions so a single sample is presented as a single slice with parasaggital projections to show the third dimension (Figure 4.A), and as a montage showing all the slices in the image stack (Figure 4.C). This same sample is also represented in a different way in Figure 4.D.i : 1 day *S. mutans* and *V. dispar* i. Here the image stack is collapsed from three dimensions into a single image that preserves the maximum intensity identified in the z-dimension in the new x-y image. This method shows much of the key information about biofilm morphology and permits the rapid comparison of many samples.

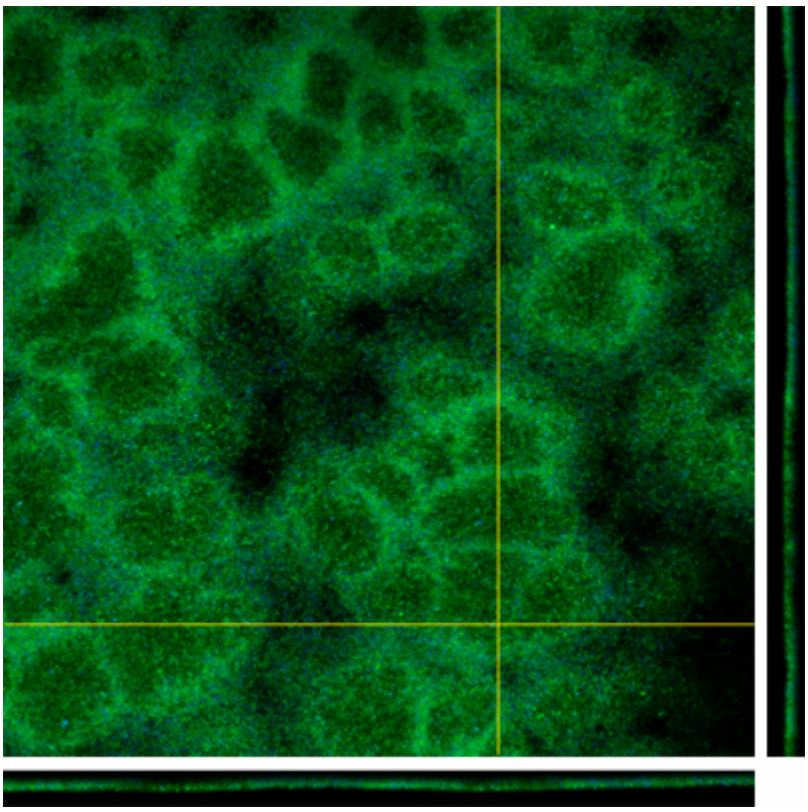


Figure 4.A. Structure of a one day dual-species *S. mutans* and *V. dispar* biofilm. This biofilm was imaged using Confocal scanning microscopy and this figure shows a single x-y frame with re-slice parasaggital projections (taken along marked yellow lines showing single x-z and y-z slices). Live bacteria are shown as green, dead bacteria are shown as blue and areas where there were both live and dead bacteria are shown as cyan. The x-y plane is 300µm x 300µm. When viewed at higher magnification, each pixel fluorescing at this magnification was found to be a bacterium or a pair or clump of bacteria.

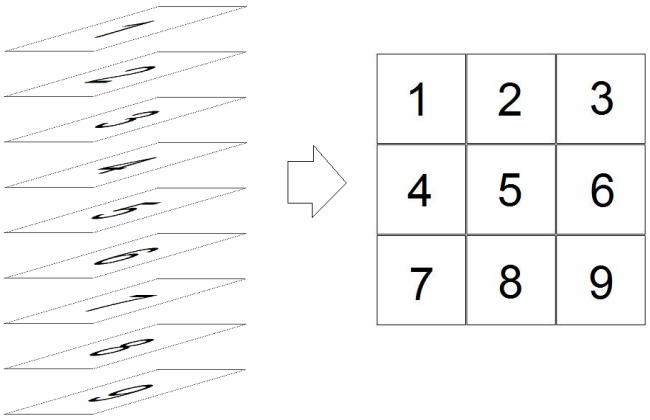


Figure 4.B. Making a montage picture from a Z-series. A Z-stack of XY raster scans is laid out to aid comparison within and between images.

Figure 4.C. Montage of the same one day dual-species *S. mutans* and *V. dispar* biofilm.

Live bacteria are shown as green, dead bacteria are shown as blue, and areas where there are both live and dead bacteria are shown as cyan. Figure 4.A shows the 11th slice of this montage which is boxed here in yellow. Each slice in the montage is 300µm x 300µm.

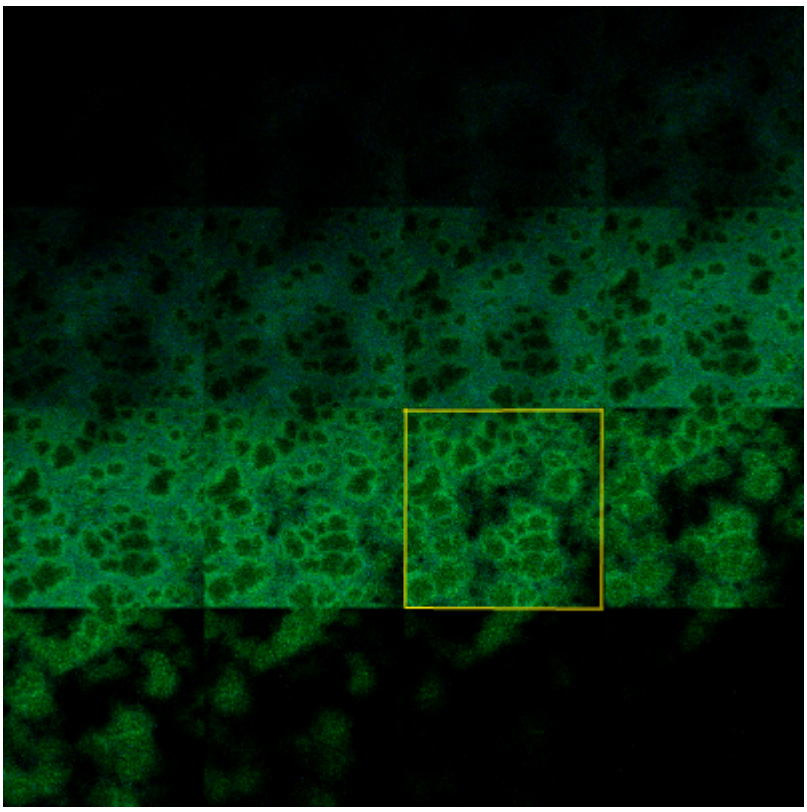


Figure 4.D, which extends over four pages, shows four different samples of each of the three biofilm types investigated for biofilms grown over one, three, seven and fourteen days. In total it shows processed images created from 48 images stacks, which is why the method of collapsing the stack but preserving the maximum intensity of the Z-dimension in the x-y image was developed. It is interesting to note the diversity of structural phenotypes that formed. This figure shows the variation within a biofilm type after a certain period of growth and the similarities and differences between the biofilm types and biofilms grown for different lengths of time. The same settings for laser intensities, gain and aperture were used while collecting the image stacks to allow direct comparison of the values, resulting in a compromise between getting good images of the one-day-old biofilms which had few bacteria and not over-exposing the images of the older biofilms which had many bacteria. There were no interstitial voids in the z-axis of any of the biofilms.

In the one-day-old biofilms, the images are mostly green indicating the bacteria are mostly viable. However one sample differed from this [Figure 4.D.i : 1 day *S. mutans* ii] as it consisted of very few bacteria and the majority of these were non-viable. In Figure 4.D.i : 1 day *S. mutans* ii the non-viable bacteria are probably due to increased processing time (double booking of the confocal microscope) causing some

of the bacteria to die rather than because the bacteria were dead after only one day of growth. In the fourteen-day-old biofilms there were more bacteria and many more of these bacteria were non-viable, and for many samples the collapsed image is saturated with the biofilm being represented as a solid block of cyan (both viable and non-viable bacteria).

There were clear differences within time points but between biofilm types. There were very few non-viable bacteria in three-day-old *V. dispar* biofilms but some to many non-viable bacteria in three-day-old *S. mutans* biofilms. Dual-species three-day-old biofilms were intermediate in vitality. In fourteen-day-old dual-species biofilms, the samples contained both viable and non-viable bacteria over much of the region but there were many small regions of just viable bacteria in all four samples, while this was not the case for any of the fourteen-day *S. mutans* and three of the four *V. dispar* biofilms; which contained a mixture of live and dead bacteria throughout the sample.

S. mutans samples for all time points tended to be rather regular, confluent biofilms. *V. dispar* appears to show two different patterns of growth; either many small but interconnected colonies (e.g. Figure 4.D.i : 1 day *V. dispar* iv) or almost the inverse of this where there is a regular lawn over much of the area but areas of no growth with a few bacteria in the centre of these, indicating they are producing inhibitory products or have depleted required nutrients.

The dual-species biofilms show much less regular patterns of growth, which is to be expected given they have two different species growing within them. They display voids, areas containing no bacteria, but also what appear to be edge effects with areas of increased vitality. The dual-species biofilms look more like the *V. dispar* biofilms than the *S. mutans* biofilms for days one, three and seven but with more bacteria.

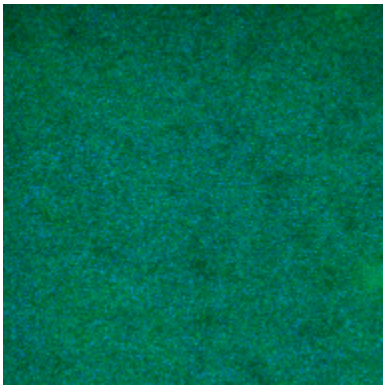
The single-species *S. mutans* biofilms show a progression of development of a confluent viable lawn after only one day, with some microcolonies within the biofilm starting to contain non-viable and viable mixes of bacteria after three days (e.g. Figure 4.D.ii : 3 day *S. mutans* iii). By seven and through to fourteen days the entire biofilm consists of an homogenous mix of both viable and non-viable bacteria.

In contrast, the single-species *V. dispar* biofilms were not all confluent after one day of growth, with some that were confluent comprising connected microcolonies separated in places by small voids containing a few bacteria (e.g. Figure 4.D.i : 1 day *V. dispar* ii and iii) and one that was not yet confluent comprising microcolonies that had almost grown to the size where the biofilm becomes confluent (e.g. Figure 4.D.i : 1 day *V. dispar* iv). After three days of growth the number of bacteria in the voids had increased (e.g. Figure 4.D.ii : 3 day *V. dispar* iv) or the microcolonies had continued to fuse, such that there were only a few small voids (e.g. Figure 4.D.ii : 3 day *V. dispar* ii and iii) or tiny voids (e.g. Figure 4.D.ii : 3 day *V. dispar* i). By seven days, the first non-viable bacteria had become evident, either disjunct from the viable bacteria at the margins of the microcolonies (e.g. Figure 4.D.iii : 7 day *V. dispar* i) or homogenously mixed with the viable bacteria through the biofilm except for some patches that were solely viable bacteria (e.g. Figure 4.D.iii : 7 day *V. dispar* ii and iii). By fourteen days the biofilms were either an homogenous mix of viable and non-viable bacteria (e.g. Figure 4.D.iv : 14 day *V. dispar* i and iv) or many distinct microcolonies of mixed viable and non-viable bacteria in a lawn of solely viable bacteria (e.g. Figure 4.D.iv : 14 day *V. dispar* ii and iii).

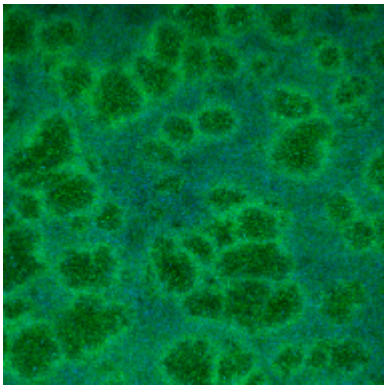
The dual-species biofilms were much more irregular. They formed confluent biofilms from one day of growth comprising a dense mosaic that consisted of bacterial lawns with regions containing microcolonies that did not have clear margins (e.g. Figure 4.D.i : 1 day *S. mutans* and *V. dispar* ii) or had clear margins that had increased density (e.g. Figure 4.D.i : 1 day *S. mutans* and *V. dispar* i). The one-day dual-species biofilms had a small number of non-viable bacteria evident but by three days of growth the biofilms comprised many non-viable bacteria (except Figure 4.D.ii : 3 day *S. mutans* and *V. dispar* iii). There were still microcolonies comprising solely viable bacteria but these existed in a lawn of mixed viable and non-viable bacteria (e.g. Figure 4.D.ii : 3 day *S. mutans* and *V. dispar* i). By seven and continuing on to fourteen days there were many more non-viable bacteria in the dual-species biofilms, comprising lawns of homogenously mixed viable and non-viable bacteria and in these there were microcolonies that were either solely viable (e.g. Figure 4.D.iii : 7 day *S. mutans* and *V. dispar* ii) or a dense mixture of both viable and non-viable bacteria (e.g. Figure 4.D.iii : 7 day *S. mutans* and *V. dispar* iv).

Figure 4.D. Confocal micrographs of single and dual-species *S. mutans* and *V. dispar* biofilms. The next 4 pages show confocal micrographs of *S. mutans* single-species biofilms, *V. dispar* single-species biofilms and *S. mutans* and *V. dispar* dual-species biofilms. Four biofilms are shown of each biofilm type for four different growth periods (one, three, seven and fourteen days), totalling 48 biofilms. In this figure considerable variation is seen between biofilms of the same type and age, between biofilm types and between different days. The reasons for the considerable variation between biofilms of the same type and age are unknown but may include differences in processing time, the natural variability of bacteria and slight variations in the environment that result in larger differences in biofilm morphology.

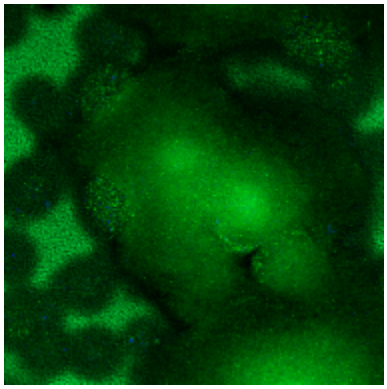
Each flattened stack shows a 300μm x 300μm area. Live bacteria are shown in green, non-viable bacteria are shown in blue and areas where both live and non-viable bacteria were detected are shown in cyan.



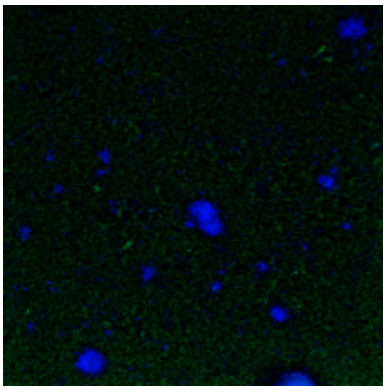
1 day *S. mutans* i



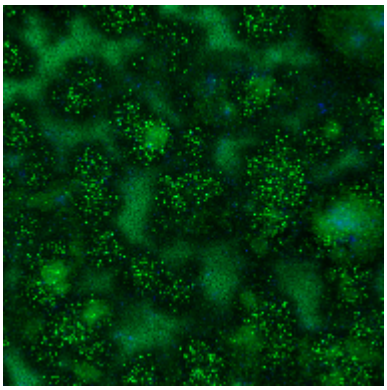
1 day *S. mutans* and *V. dispar* i



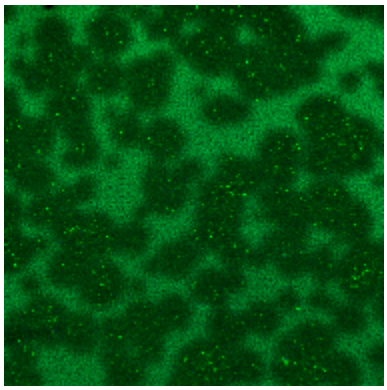
1 day *V. dispar* i



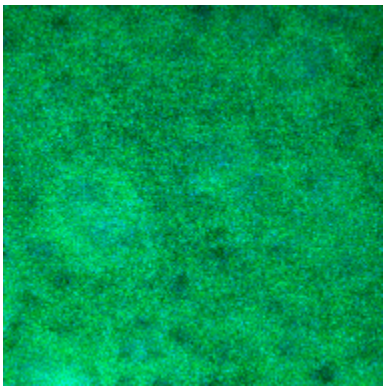
1 day *S. mutans* ii



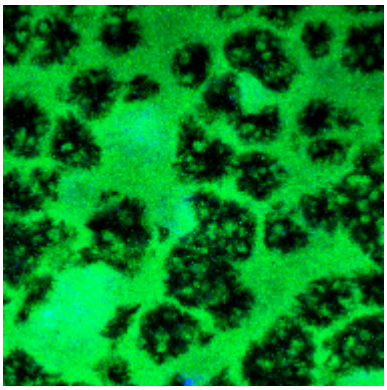
1 day *S. mutans* and *V. dispar* ii



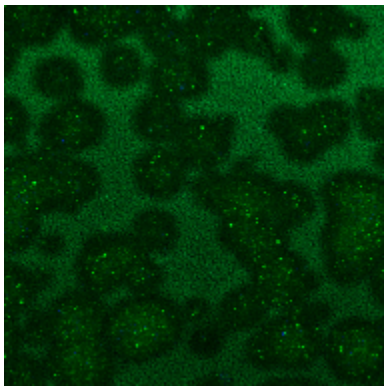
1 day *V. dispar* ii



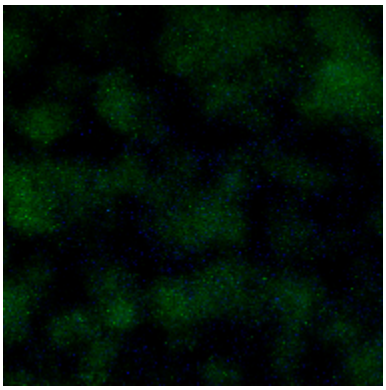
1 day *S. mutans* iii



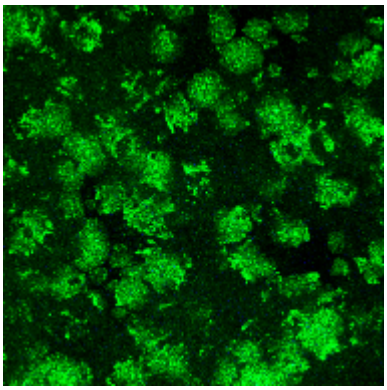
1 day *S. mutans* and *V. dispar* iii



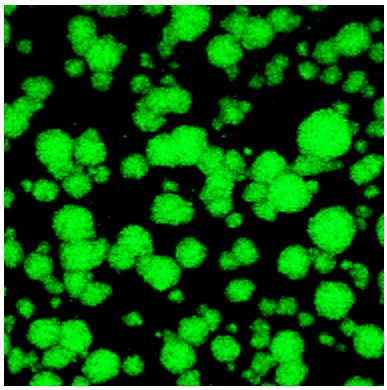
1 day *V. dispar* iii



1 day *S. mutans* iv

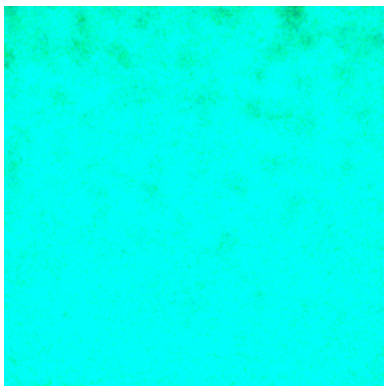


1 day *S. mutans* and *V. dispar* iv

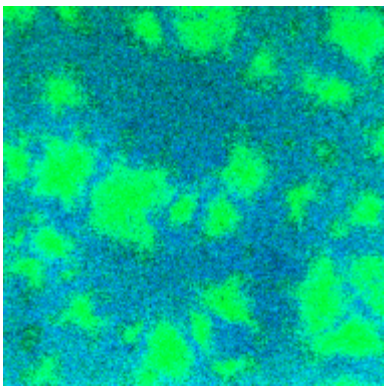


1 day *V. dispar* iv

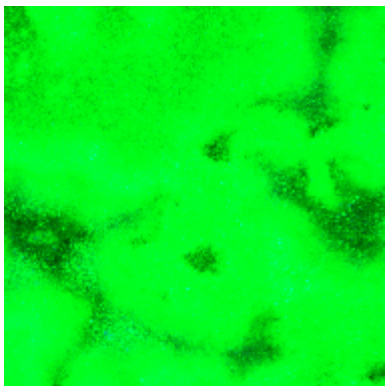
Figure 4.D.i. One day biofilms.



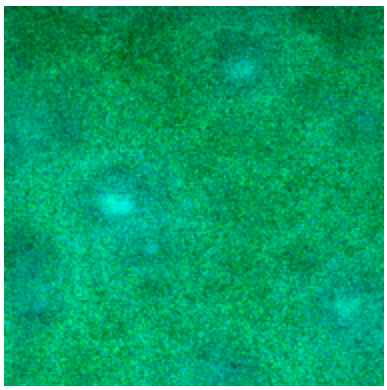
3 day *S. mutans* i



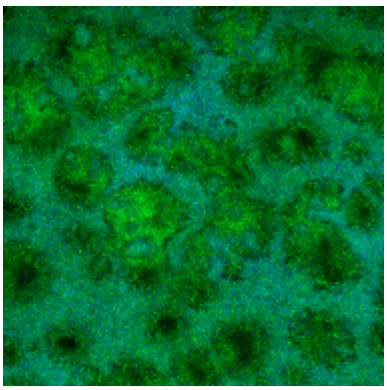
3 day *S. mutans* and *V. dispar* i



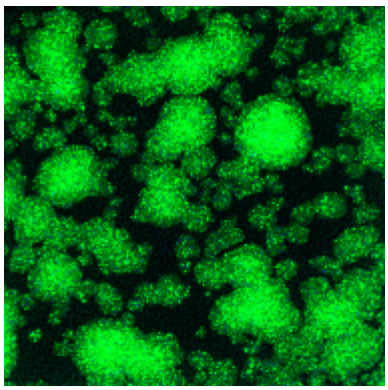
3 day *V. dispar* i



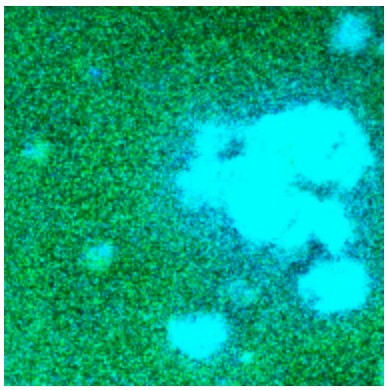
3 day *S. mutans* ii



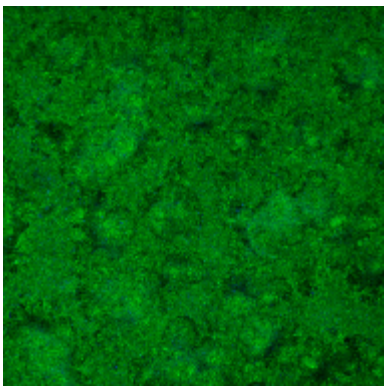
3 day *S. mutans* and *V. dispar* ii



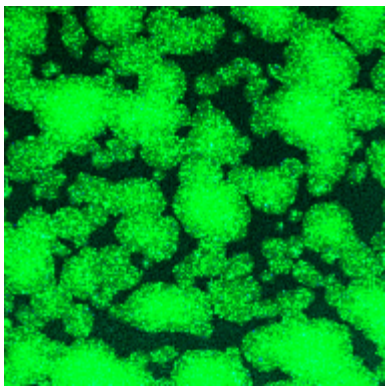
3 day *V. dispar* ii



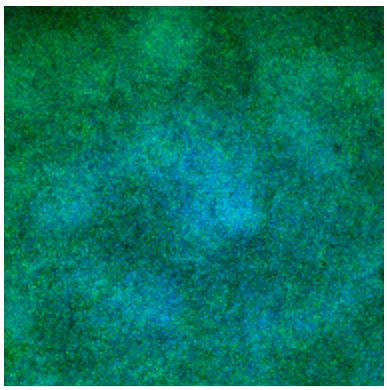
3 day *S. mutans* iii



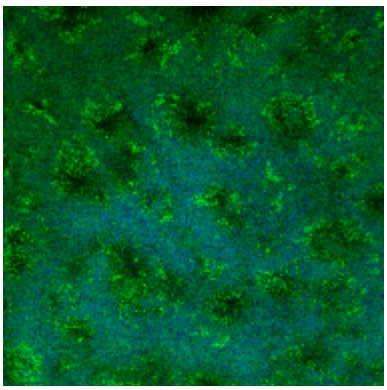
3 day *S. mutans* and *V. dispar* iii



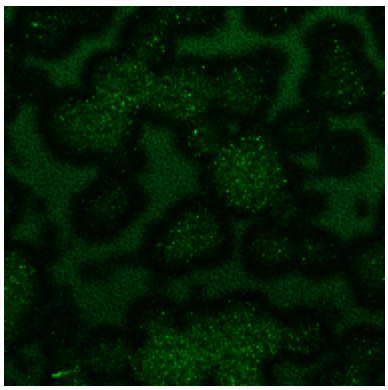
3 day *V. dispar* iii



3 day *S. mutans* iv

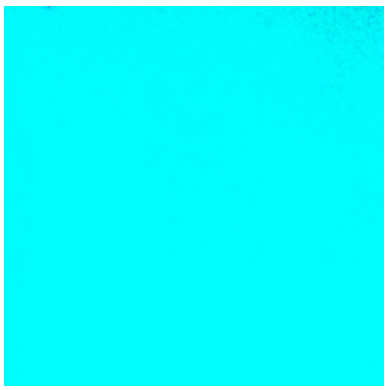


3 day *S. mutans* and *V. dispar* iv

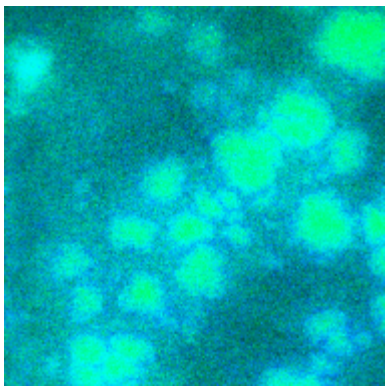


3 day *V. dispar* iv

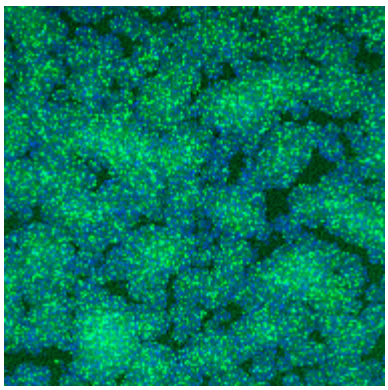
Figure 4.D.ii. Three day biofilms.



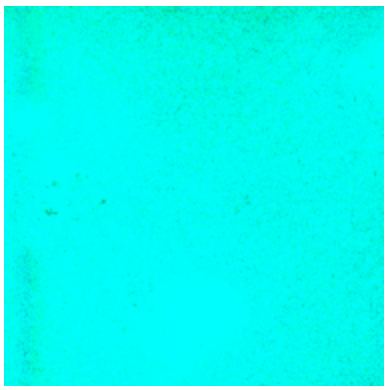
7 day *S. mutans* i



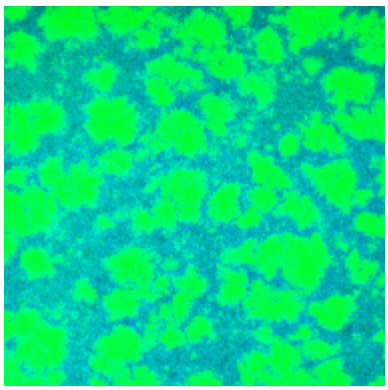
7 day *S. mutans* and *V. dispar* i



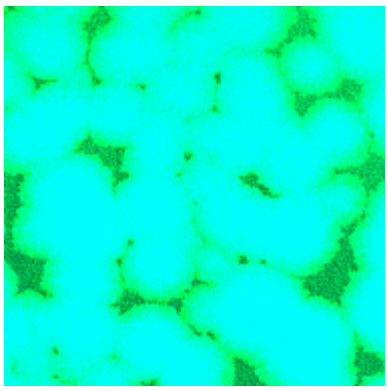
7 day *V. dispar* i



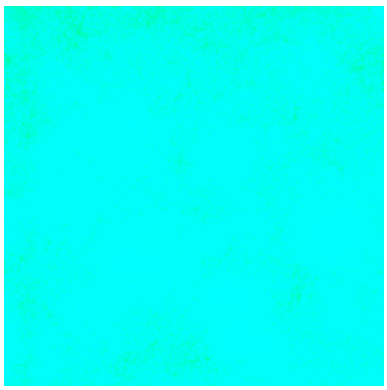
7 day *S. mutans* ii



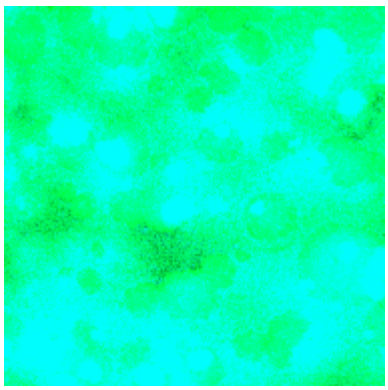
7 day *S. mutans* and *V. dispar* ii



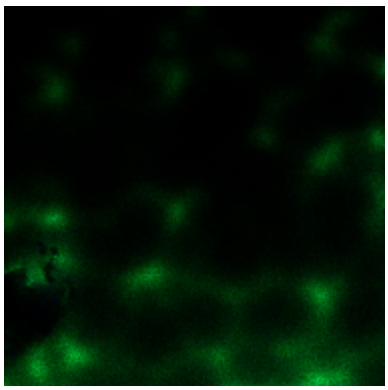
7 day *V. dispar* ii



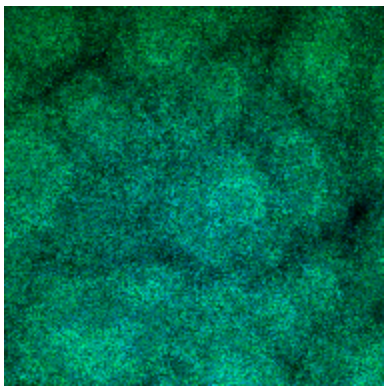
7 day *S. mutans* iii



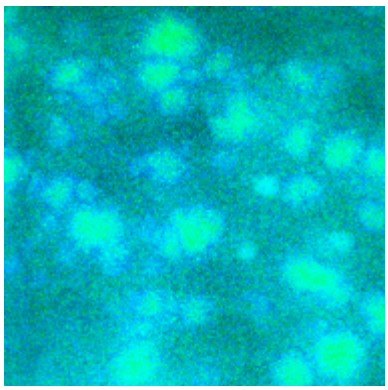
7 day *S. mutans* and *V. dispar* iii



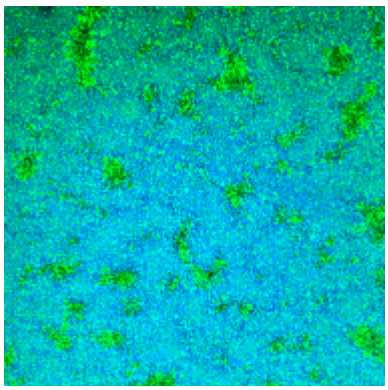
7 day *V. dispar* iii



7 day *S. mutans* iv

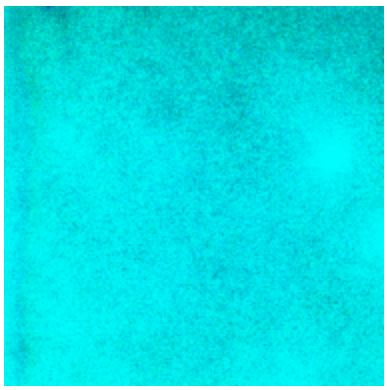


7 day *S. mutans* and *V. dispar* iv

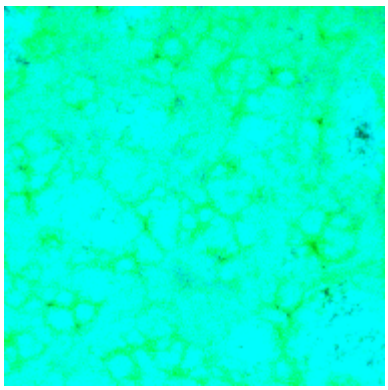


7 day *V. dispar* iv

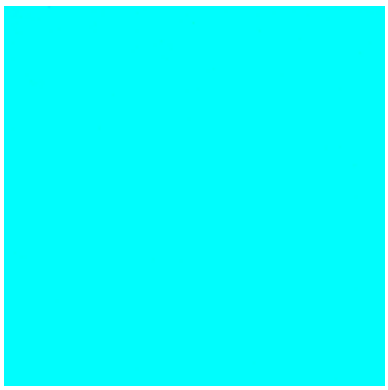
Figure 4.D.iii. Seven day biofilms.



14 day *S. mutans* i

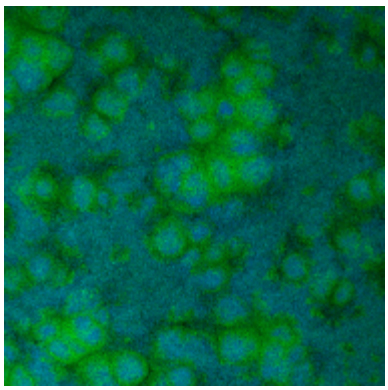


14 day *S. mutans* and *V. dispar* i

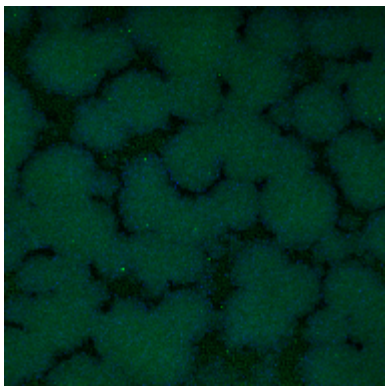


14 day *V. dispar* i

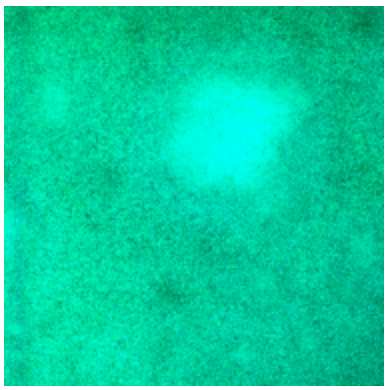
14 day *S. mutans* ii



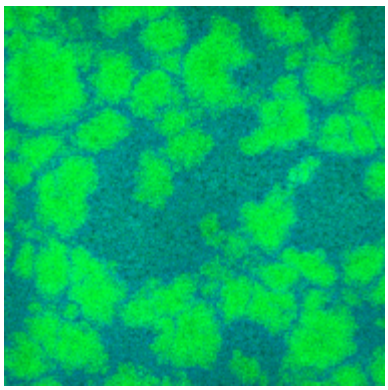
14 day *S. mutans* and *V. dispar* ii



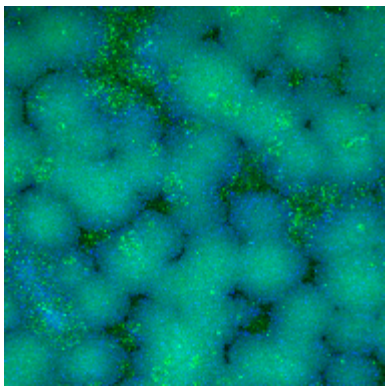
14 day *V. dispar* ii



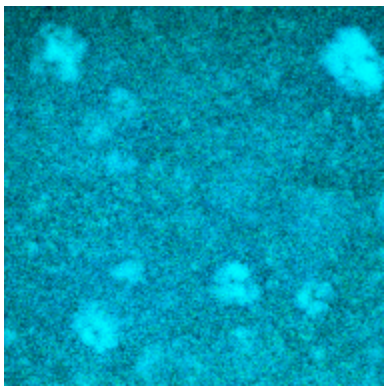
14 day *S. mutans* iii



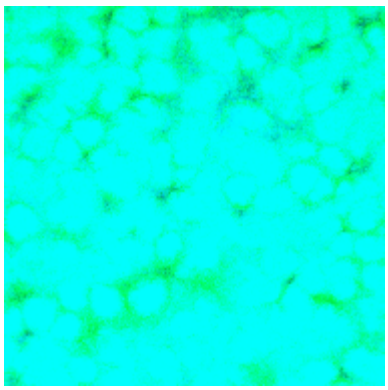
14 day *S. mutans* and *V. dispar* iii



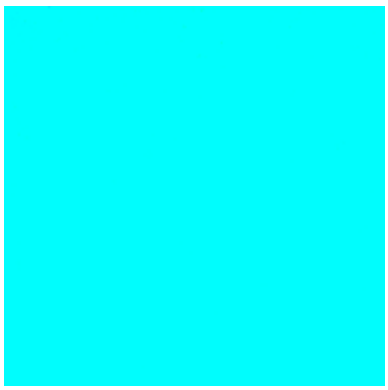
14 day *V. dispar* iii



14 day *S. mutans* iv



14 day *S. mutans* and *V. dispar* iv



14 day *V. dispar* iv

Figure 4.D.iv. 14 day biofilms.

Table 4.A presents summary statistics of the results of the image analysis of the biofilms. The intensity of the viable (green) channel was almost always higher than the intensity of the non-viable (blue) channel and the values of both increased over time for all biofilm types.

Table 4.A. Image analysis of biofilms samples scanned using confocal microscopy.

Summary statistics of vitality and thickness of the 48 biofilms shown in Figure 4.D. Viable bacteria fluoresce green and dead bacteria fluoresce red. Fluorescence is unitless and takes values between 0 and 253. Thickness is the thickness of the 300μm x 300μm image stack required to sample the biofilm and not the thickness of the biofilm (most biofilms were approximately 20μm thick at any vertical line of xy points but the biofilm did not align with the xy plane and consequently the deepest point at one part of the scan was often higher than the shallowest point at another part of the scan).

	average green fluorescence (live)	standard deviation of green fluorescence (live)	average red fluorescence (dead)	standard deviation of red fluorescence (dead)	Average thickness (μm)	standard deviation thickness (μm)
1 day *S. mutans*	73.5	69	44.6	41.8	35.5	13.4
1 day *S. mutans* and *V. dispar*	89.9	36.4	24.7	21	53.5	16.1
1 day *V. dispar*	68.8	15	8.2	5.5	39.5	16.6
3 day *S. mutans*	174.2	56.8	140.4	66.6	50.5	12.2
3 day *S. mutans* and *V. dispar*	118.5	41.4	59	59.1	44.0	6.5
3 day *V. dispar*	129.8	90.2	19.9	12.7	46.5	39.8
7 day *S. mutans*	215.4	73	202.4	86.1	62.0	31.4
7 day *S. mutans* and *V. dispar*	206.2	36.6	164.3	24.6	46.0	11.2
7 day *V. dispar*	140.5	97.2	116.5	89.2	39.0	26.6
14 day *S. mutans*	206.4	40.1	190.3	31	70.0	4.3
14 day *S. mutans* and *V. dispar*	194.4	74.1	153.5	85.3	53.0	22.0
14 day *V. dispar*	169.2	102	162.3	109.9	50.5	8.5

The factors affecting the fluorescent intensity caused by dead bacteria were investigated using a general linear model. The model was highly significant ($p<0.001$) and had an adjusted R^2 of 0.531. Days of growth was highly significant ($p<0.001$)

[fourteen day old biofilms had more dead bacteria than one and three day old biofilms; Dunnett's two-sided t-tests, $p<0.001$ and $p=0.001$ respectively], as was the effect of biofilm type ($p=0.01$) [*S. mutans* biofilms had more dead bacteria than *V. dispar* biofilms; Dunnett's two-sided t-test, $p=0.005$]. Dual species biofilms did not differ significantly from either of the types of single species biofilms.

The factors affecting the fluorescent intensity caused by live bacteria were investigated using a general linear model. The model was highly significant ($p<0.001$) and had an adjusted R^2 of 0.309. Days of growth was the only significant factor and it was highly significant ($p<0.001$) [both seven and fourteen day old biofilms had more live bacteria than one day old biofilms; Dunnett's two-sided t-tests, $p<0.001$ for both].

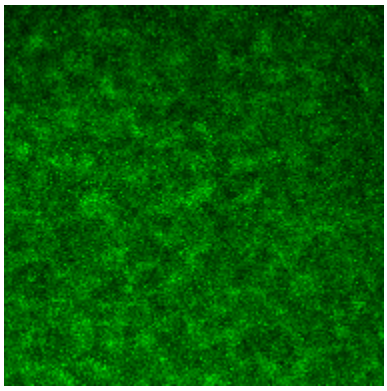
The depth of the biofilms was investigated using a general linear model. The model fitted the data poorly (adjusted $R^2 = -0.12$) and was not significant ($F=0.949$, d.f.=11, $P=0.507$) indicating there was no effect of day or species composition on the depth of the biofilm (univariate analysis of variance testing between subject effects [dependent variable: slices, factors: day, contains *S. mutans*, contains *V. dispar*]). The biofilms varied in depth between 16 and 106µm, however at any individual point in a biofilm the thickness was typically about 20µm. That is at any x-y point in an x-y-z stack, there would be live or dead bacteria on about 10 adjacent slices which were 2µm apart, while there may be many more than 10 slices in the Z-series with bacteria on them. The distance between 10 adjacent slices is 18 µm but the slices are not expected to be at the exact edges of the biofilm so the best approximation of the thickness of the biofilm is to assume the biofilm extends half way to the next point sampled in both directions, and thus the estimate of thickness for a biofilm observed over 10 slices is 20 µm [18+1+1] (an alternative explanation is that as samples are taken every 2 µm each slice represents 2 µm of thickness, thus a 10 slice biofilm is 20 µm thick). The large variation in depth of the biofilms, while being of roughly similar thickness, was due to the biofilm not being perfectly aligned with the xy plane.

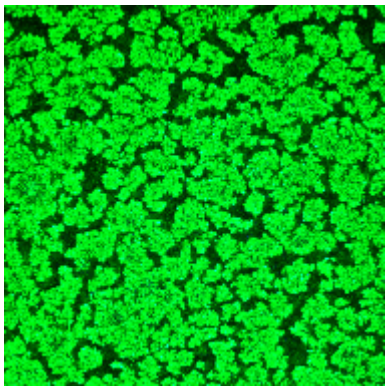
To investigate whether there was any vertical partitoning of vitality within the biofilm fluorescent intensities of the live and dead channels was plotted against the z-axis. However, as the biofilms did not align in the xy axis these plots averaged the

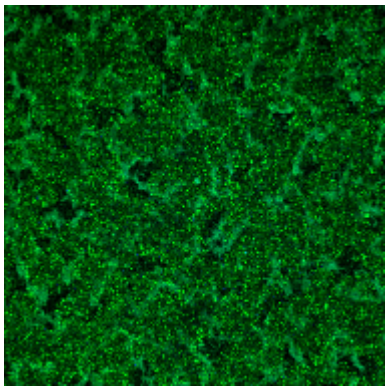
highest point of one part of the biofilm with the lowest point of a different part of the biofilm. Consequently these plots are not shown. After investigating each slice of all the image stacks it is the author's impression that there is no vertical partitioning of vitality but unfortunately this can not be tested statistically due to xy points in the same image slice being at different relative depths of the biofilm.

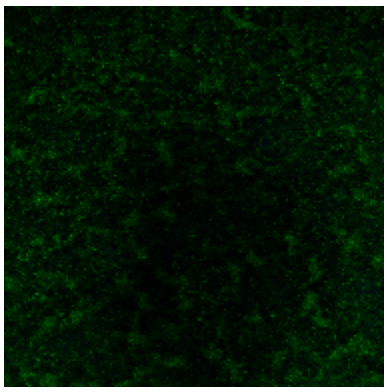
4.3.1.2 Effect of D-glucose on the structure and vitality of *S. mutans* and *V. dispar* biofilms

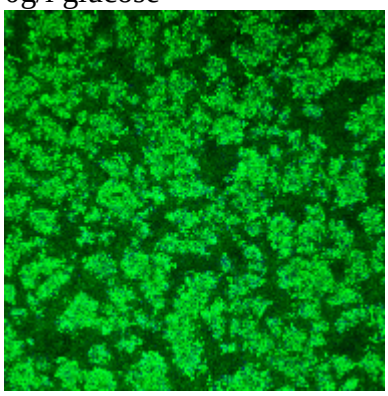
Figure 4.E shows collapsed image stacks (as with Figure 4.D) of seven day old biofilms grown using different concentrations of D-glucose.

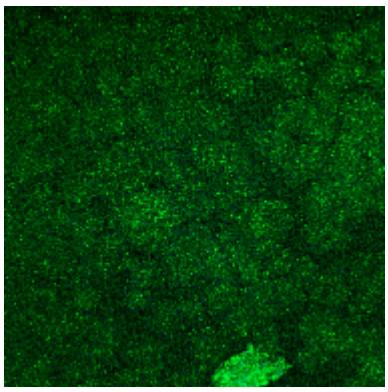

7 day *S. mutans* 0g/l glucose

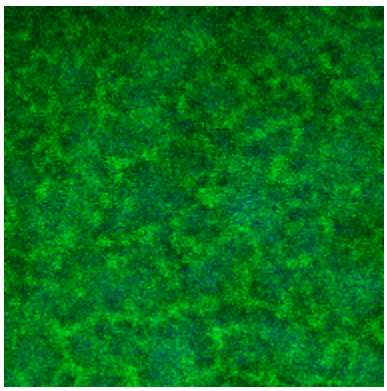

7 day *S. mutans* and *V. dispar* 0g/l glucose

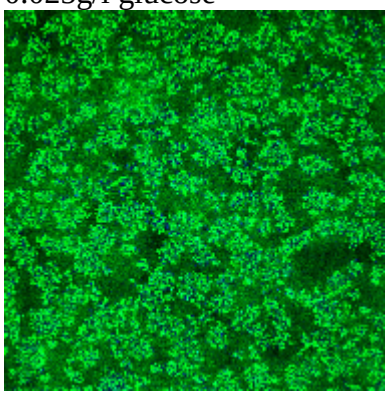

7 day *V. dispar* 0g/l glucose

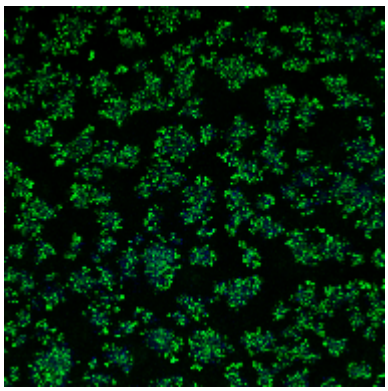

7 day *S. mutans* 0.025g/l glucose


7 day *S. mutans* and *V. dispar* 0.025g/l glucose


7 day *V. dispar* 0.025g/l glucose


7 day *S. mutans* 0.25g/l glucose


7 day *S. mutans* and *V. dispar* 0.25g/l glucose


7 day *V. dispar* 0.25g/l glucose

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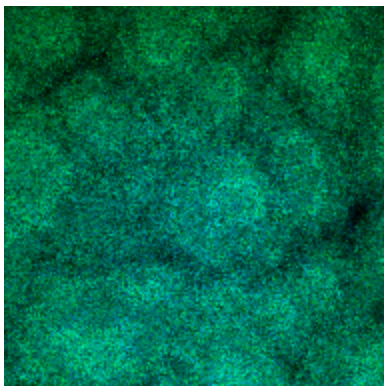
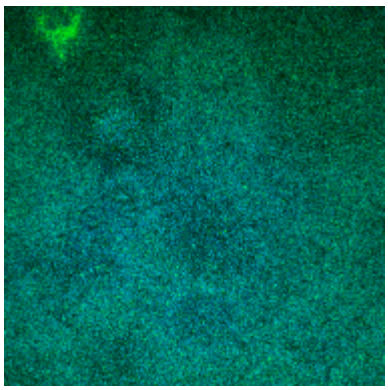
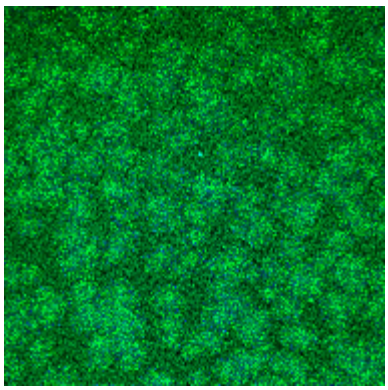


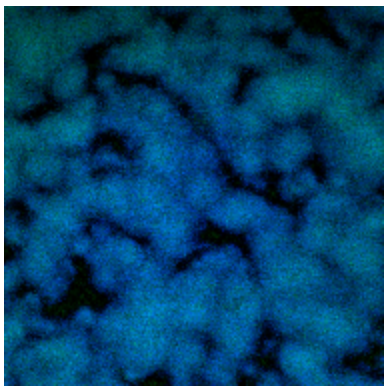
7 day *S. mutans* 2.5g/l glucose



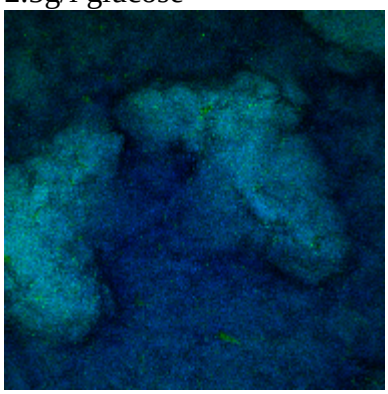
7 day *S. mutans* and *V. dispar* 2.5g/l glucose



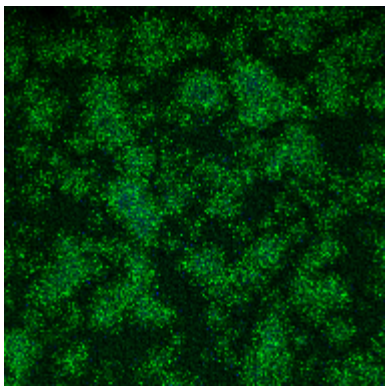
7 day *V. dispar* 2.5g/l glucose



7 day *S. mutans* 25g/l glucose



7 day *S. mutans* and *V. dispar* 25g/l glucose



7 day *V. dispar* 25g/l glucose

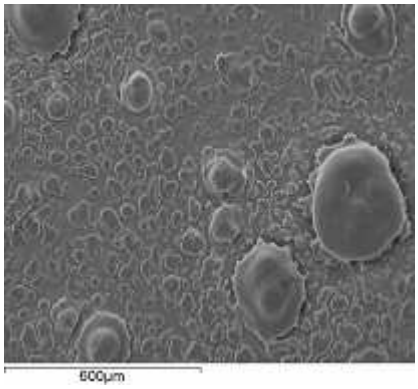
Figure 4.E. Seven day old biofilms showing the effects of glucose on growth and viability. Each flattened stack shows a 300µm x 300µm area. One representative scan is shown for each type of single-species biofilm and for each dual species biofilm for each of the glucose concentrations.

Figure 4.E shows the proportion of non-viable bacteria in all biofilms containing *S. mutans* increases as the concentration of glucose increases. The single species *V. dispar* biofilms show differences with varying glucose concentration but are similar in their composition of mostly live bacteria. In both single and dual species biofilms containing *S. mutans* there is a clear increase in the relative proportion of dead bacteria as glucose concentration increases. The biofilms which included *S. mutans* that grew on medium containing 25 g/l D-glucose consisted of predominantly dead bacteria. At lower concentrations of glucose, biofilms containing *S. mutans* thrived. In the dual species biofilms the microcolonies were crisp, tight, and tiny and comprised solely viable bacteria when no glucose was added whereas when 25g/l glucose was added the microcolonies were big and bulbous and comprised mostly non-viable bacteria.

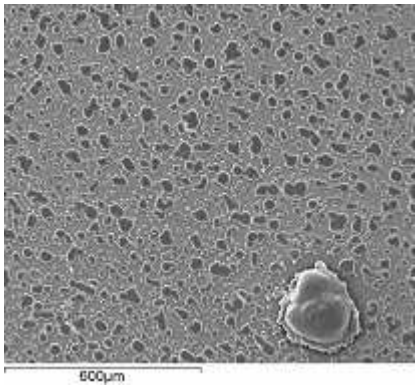
4.3.2 Scanning Electron Microscopy

Scanning electron micrographs were taken for the three types of one day biofilms and they provide some very interesting information about the biofilms (see Figure 4.F). *S. mutans* biofilms have a few very large umbonate colonies (≈300μm in diameter) and many smaller patches (≈30μm in diameter). *V. dispar* biofilms have many similar sized patches (≈10μm - 50μm in diameter). Dual species biofilms display both types of patterns. At higher magnification *S. mutans* biofilms can be seen to have many chains of bacteria piled over each other. *V. dispar* biofilms have orderly arranged bacteria that form distinct layers. In the highest magnification of the dual species biofilm (top middle micrograph) *S. mutans* (top left of micrograph) and *V. dispar* (bottom right of micrograph) are overlapping (not growing as discrete species). In this micrograph *V. dispar* also appears to be growing less regularly.

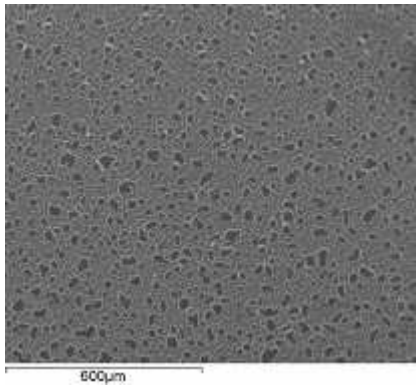
In comparison with CLSM images shown in Figure 4.D.i where nine of the twelve biofilms were confluent, not one of the three of these biofilms is confluent. The microcolonies of all three biofilm types have irregular margins; some undulate but mostly lobate margins (probably caused by the fusion of two or more smaller colonies leaving indentations adjacent to the areas where the smaller colonies fused).



S. mutans
1 day biofilm 40x

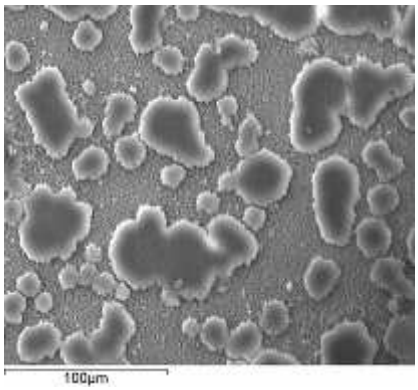


S. mutans and *V. dispar*
1 day biofilm 40x

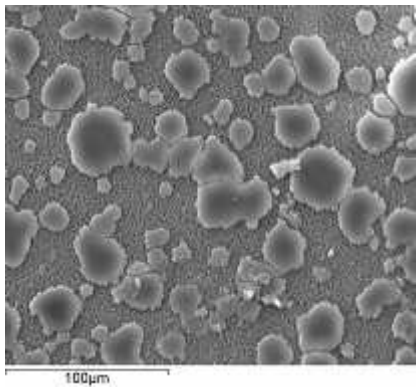


V. dispar
1 day biofilm 40x

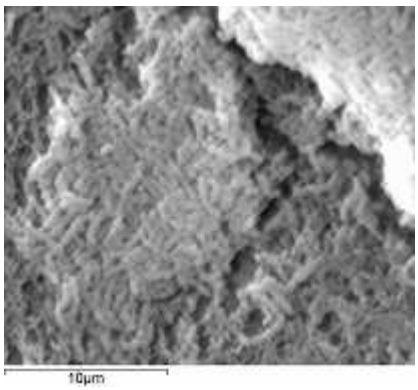
S. mutans
1 day biofilm 200x



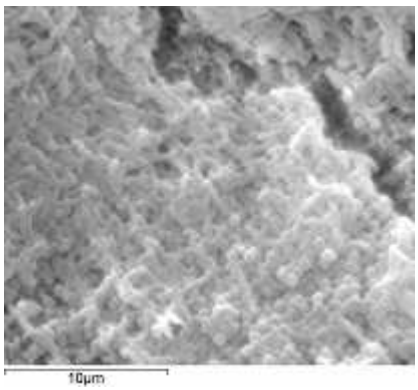
S. mutans and *V. dispar*
1 day biofilm 200x



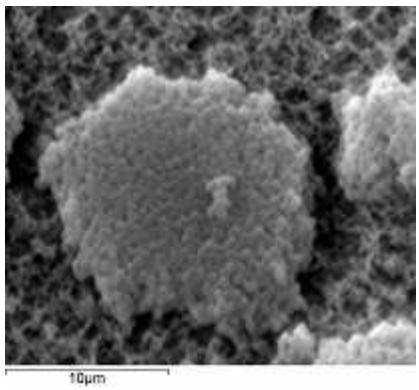
V. dispar
1 day biofilm 200x



S. mutans
1 day biofilm 2000x



S. mutans and *V. dispar*
1 day biofilm 2000x



V. dispar
1 day biofilm 2000x

Figure 4.F. Scanning Electron Micrographs of the different biofilms after one day of growth. SEM pictures at different magnifications (40X, 200X and 2000X) of the three types of biofilm.

4.3.3 Summary of Results

This chapter investigated whether there were spatial differences between single and dual species *S. mutans* and *V. dispar* biofilms.

- *S. mutans* formed regular and confluent biofilms at all time points.
- *V. dispar* displayed two different growth patterns; many small interconnected colonies or almost the reverse of this (a lawn with circular holes).
- Dual species biofilms displayed edge effects (areas of no bacteria at growth boundaries).
- 14 day biofilms had significantly more non-viable bacteria than 1 and 3 day old biofilms.
- *S. mutans* biofilms comprised more non-viable bacteria than *V. dispar* biofilms.
- Dual species biofilms did not differ significantly from either of the types of single-species biofilms.
- There were no effects of length of growth or species composition on the depth of the biofilms.
- Most mature biofilms were approximately 20μm thick.
- There were no interstitial voids in the z-axis of any of the biofilms.
- The number of non-viable bacteria increased as the concentration of glucose increased in all seven-day-old biofilms containing *S. mutans*.
- A glucose concentration of 25g/L resulted in predominantly non-viable bacteria in any biofilms comprising *S. mutans*.
- *S. mutans* formed crisp and viable colonies at low glucose concentrations and large, bulbous and non-viable colonies at high glucose concentrations.
- *S. mutans* had a few large, umbonate colonies (≈300μm) and many smaller (≈30μm) colonies in one-day-old colonies viewed using SEM.
- *V. dispar* had many smaller (≈10-30μm) colonies (1 day SEM).
- The dual species biofilms had a mixture of the colonies observed for the two types of single species biofilms (1 day SEM).
- *S. mutans* was observed to form chains of bacteria piled over each other while *V. dispar* formed very ordered layers of bacteria (1 day SEM).
- One-day-old dual-species biofilms typically comprised many small adjacent patches of a single species of bacteria, but occasionally these patches over-

lapped and both species of bacteria were in direct physical contact with the other species (1 day SEM).

4.4 Discussion

To control the development of biofilms it is important to understand the process of how single cells that attach to a surface develop into complex biofilms (Klausen et al. 2003). The standard view of biofilms is of microcolonies dispersed in a matrix with fluid channels running through to provide nutrients and remove waste (reviewed in Costerton et al. 1995). The biofilm growth model used in this study differed from typical oral biofilms and from commonly used biofilm models in that nutrients came through a permeable but unencroachable substrate and the biofilm was not in bulk fluid and thus there was less benefit to having circulatory channels throughout the biofilm as there was less fluid to flow through them (the biofilm was still immersed in liquid as a consequence of capillary action from the media but there was less liquid than in oral biofilms and commonly used models). Consequently it is interesting to see how the biofilms in this study develop and what structural features they display.

Vroom et al. (1999) found extreme variation in pH can develop in biofilms in response to the addition of sucrose such that microcolonies with a pH of less than 3.0 could exist adjacent to areas with a pH of greater than 5.0. Without a circulatory network there is even more potential for vastly different microenvironments to develop. Furthermore, the available nutrients and the end products that are present locally within a biofilm depend partially on neighbours (Costerton et al. 1995) but in these biofilms this dependence could be even more extreme. However, despite the possibility for extremely different microenvironments this growth model presented, the biofilms were surprisingly homogenous with no stratification of vitality at different depths although this could not be tested statistically.

There were interesting structures particularly at the margins of the microcolonies but over time most biofilms developed into dense mats of bacteria that were about 20µm thick. This section comments on the structure and growth of these biofilms and compares the results found with those in the literature. It expands specifically on the depth and thickness of the biofilms, their vitality, what these findings potentially mean for controlling these biofilms and what avenues have potential for further study in this system.

4.4.1 Structure and Growth

The biofilm growth model used in these experiments is very simple as nutrients diffuse from the substrate (through the filter) and the bacteria are growing in gas rather than liquid. Complex structures may form either as a consequence of the environment, such as growth limited by nutrient transfer resulting in channels and voids (Picioreanu et al. 1998) or by design, where genes control the spatial organisation of the bacteria (De Kievit et al. 2001, Stoodley et al. 2002) and this growth model (the environment) has resulted in very dense biofilms.

There are clear differences in the structure of the different biofilms and clear changes over time. The Scanning Electron micrographs showed how the method of fission of the bacteria directly influences the microstructure of the biofilms as many randomly oriented chains of bacteria are evident in *S. mutans* biofilms (due to bacteria always dividing in the same plane) in contrast to the sheets of bacteria which were evident in the *V. dispar* biofilm (which result from the 90 degree rotations in the plane of division). The Scanning Electron micrographs also showed a few very large *S. mutans* microcolonies (considerably larger than the other microcolonies of either species) which were probably due to clumps of bacteria being inoculated on to the filter as a result of the strong attachments between the bacteria in the chains. It is interesting to see evidence of the production of inhibitory molecules (or possibly the localised depletion of nutrients) in some of the one and three day old single species biofilms. This is despite the high relatedness of the bacteria because the biofilms were inoculated from the same culture. Both of the species used in this study are non-motile so when the filters were inoculated with the bacteria at the start of each experiment, once the bacteria attached the structure depended upon how the bacteria grew from there. In contrast to this, Klausen et al. (2003) investigating *P. aeruginosa* biofilms found that bacteria would migrate to form the caps of mushroom like structures.

Much like colonial growth, the biofilms in this study grew through a combination of leading edge growth until the biofilm was confluent and diffusion limited growth in the central areas of the microcolonies and diffusion limited growth over the entire biofilm once it was confluent (Wimpenny 1979). In agreement with the findings of this study, where there were initially visible gaps between microcolonies before the biofilms became confluent, Dalwai et al. (2006) found an open structure

with visible voids changing to a tightly packed structure with fewer voids as the biofilm developed. Briandet et al. (2008) found that despite growing in a flow cell (with obvious potential for flow), *Lactobacillus lactis* grew as a dense biofilm with no voids, similar to the structure of many of the biofilms imaged in this study (while other species Briandet et al. [2008] studied grew in the standard well described way with channels and voids).

The total viable counts of the biofilms showed that each species does not appear to be limited by the presence of the other bacterium. This is surprising as the expectation would be that space would be a limiting factor. This investigation has not shown dual species biofilms to be more densely packed or thicker despite the fact they were comprised of more bacteria.

Although after even only one day of growth many of the biofilms were already confluent, *V. dispar* single species biofilms showed a transition of microcolony growth to confluent biofilms over the time points scanned. This is because *V. dispar* grows slower than *S. mutans* which would have benefited from scanning at earlier time points as well to better understand the earlier stages of *S. mutans* biofilm development. As the microcolonies grew, prior to becoming confluent, there would have been less restricted growth (less inhibition due to waste and better access to nutrients) at the leading edges of the biofilm (similar to bacterial colonies Wimpenny 1979) however in single species *V. dispar* biofilms there were indications of inhibitory products possibly being produced as there was less growth at the margins (of some, but not all, biofilms). If multiple scans of the same biofilm were taken, including earlier time points, it would be possible to determine if rims (the margins at the leading edge) were growing differently to hubs (the bulk of the microcolonies).

An interesting difference between the biofilms is that some have sparse lawns of bacteria with dense microcolonies scattered throughout them while others do not. There are no very early time points to determine whether this is due to the entire lawn being seeded but also a few clumps of bacteria attaching and then becoming microcolonies, the entire lawn being seeded and some bacteria growing much faster and thus becoming microcolonies or an unknown method of *S. mutans* dispersing into the voids to create the lawns. Access to nutrients will be improved in sparsely populated lawns compared to access available in microcolonies (excluding nutrients

that are waste products of other bacteria) yet *Streptococcus* spp. and *Veillonella* spp. are known to cluster (Egland et al. 2004, Kara et al. 2007) and this clustering could be responsible for increased resistance to chlorohexidine in dual species biofilms (Kara et al. 2007).

Dense biofilms do not provide increased resistance to phages (Briandet et al. 2008) or antimicrobials (Hope and Wilson 2004) in single or multispecies colonies, as rates of diffusion are fast, even through dense biofilms (Stewart 2003, Hope and Wilson 2004). However Stewart (2003) shows a confocal image of an *S. mutans* biofilm with surviving cores of viable bacteria encased in a non-viable outer layer after exposure to antimicrobial agent (unspecified mouthwash) and suggests it is unlikely the antimicrobial failed to penetrate but that the clustering of viable bacteria in the interior may still be due to diffusion, but as a result of nutrient limitation inducing a more resistant growth phase or quorum sensing (with increased signal in the interior) inducing the expression of protective genes.

The *S. mutans* biofilms in this study were confluent after three days of growth but never grew much thicker than 20µm (at any vertical point in the plane) in contrast to results of another study that employed the same model of growth where *S. sanguinis* formed biofilm clusters with defined edges (were not confluent) after three days of growth and formed thick (90µm) microcolonies after overnight growth (Bryce et al. 2009). Waite et al. (2005) found a confluent *P. aeruginosa* biofilm formed after only 14 hours, after which it did not change structurally when scanned at one and two days of growth, which, although different time points were scanned, appears consistent with the findings of this study.

4.4.2 Depth and Thickness of the Biofilms

Surprisingly, the biofilms were all about 20µm thick despite varying in depth (the range over which the whole biofilm had to be sampled to totally sample the y-profile of the 300µm by 300µm area) from 19 to 106µm. This constraint is probably due to limitations of growth set by diffusion of nutrients in the vertical axis. However, confocal microscopy has been shown to be limited in the depth of sample it can visualise while maintaining contrast and signal (Vroom et al. 1999) yet the limit is about 40µm and a similar study on nitrocellulose filters recorded depths of biofilms

of 90µm (Bryce et al. 2009). Furthermore, retarded diffusion of BacLight stain into dense biofilms has been reported (Bryce et al. 2009). However, it is likely this threshold of 20µm is genuine rather than artefactual as it is considerably under the 40µm reported by Vroom et al. (1999) even allowing for possible differences in laser type and power. The differences in depth are probably due to the alignment of the nitrocellulose filter not always matching the alignment of the x-y plane of the microscope.

Increasing thickness of the biofilm allows the development of gradients in pH and nutrients yet, despite the threshold mentioned previously, it appeared there was no stratification in viability distributions. However, even anaerobic oral bacteria are not particularly stratified (at basal areas of the biofilm away from oxygen) in natural plaque biofilms (Bradshaw et al. 1996, Watson and Robinson 2005). Increased nutrients generally results in denser biofilms (Sutherland 2001) yet in the glucose concentration experiment this was not observed, presumably because the lactic acid produced cancels out the benefits, except for the potential for the biofilm to grow very rapidly. Kara et al. (2007) found in a similar biofilm pairing that all biofilms increased in thickness over time which was not the case in this study as all biofilms grew to about 20µm.

4.4.3 Vitality of the Biofilms

As mentioned in the last section, there was no stratification of viable and non-viable bacteria despite the polarised supply of nutrients. Dalwai et al. (2006) found that the relative amount of non-viable bacteria increased closer to the surface after the addition of GCF (simulating the effect of gingivitis) whereas in this study it appeared the distribution of viable and nonviable bacteria remained relatively constant throughout the thickness of the biofilm (even though the numbers of both increased). Auschill et al. (2001) reported oral biofilms that were predominantly non-viable bacteria with clusters of viable bacteria throughout while Hope and Wilson (2003) found in some of their biofilms the opposite of this, where viable bacteria were distributed evenly throughout the biofilm with individual non-viable bacteria distributed evenly throughout the biofilm. In this study biofilms, once they started to contain non-viable bacteria, tended to be a homogenous lawn of viable and non-viable bacteria and microcolonies that were either dense clumps of viable bacteria or

dense clumps of viable and non-viable bacteria. This study found more non-viable bacteria at the later time points, with single species *S. mutans* having more non-viable bacteria than single species *V. dispar* biofilms. Dual species biofilms did not differ significantly from single species biofilms in the number of non-viable bacteria which is in contrast to Kara et al. (2007) who found dual species biofilms had relatively decreased vitality in the dual species biofilms they studied. Kara et al. (2007) postulated that this may be due to a faster life-cycle resulting from the metabolic interactions of the two species. However this study found no difference in vitality between single and dual species biofilms.

The images of the biofilms growing with different glucose concentrations available to them suggest that if glucose is present, *S. mutans* will metabolise it into lactic acid and keep metabolizing it until doing so kills it. It is also interesting to see large numbers of viable bacteria and very few non-viable bacteria when *S. mutans* grows in the absence of glucose or when it is provided with limited amounts of glucose.

4.4.4 Implications for Control of these Biofilms

A key feature of these biofilms is how dense they are, and this would reduce the penetration of nutrients. The channels found in many biofilms facilitate the acquisition of nutrients and removal of waste throughout the biofilm. Modelling and experimental studies have shown limited inhibition of penetration of antimicrobials into dense biofilms (Stewart 1996, Stewart 2003, Hope and Wilson 2004) but the dense packing may result in changes in cell state due to nutrient limitation, or quorum sensing inducing expression of protection genes that cause these dense biofilms to be harder to treat with antibiotics (Stewart 2003). Microsensor measurements of metabolic activity through a biofilm, after treatment with chlorohexidine, show reduced viability in surface bacteria but that there was little change in metabolic activity deeper within the biofilm (von Ohle et al. 2010). Pratten et al. (2000) found the addition of sucrose can lead to denser biofilms with fewer voids that are more uniform, which are similar to the biofilms in this study but they also note that this type of biofilm structure has been implicated in the development of caries.

4.4.5 Future work

Analysis programs that can perform pattern recognition and can quantify biofilm volume have been developed (e.g. COMSTAT [Heydorn et al. 2000] and DAIME [Daims et al. 2006]) but these work better with biofilms growing at a solid-liquid interface however with some work they could be used to further quantify this set of biofilms.

This study distinguished between live and non-viable bacteria rather than between species, but with access to a three laser confocal microscope, a similar kit to the BacLight Live/Dead kit, the ViaGram kit could be used which not only indicates viability but it also selectively stains Gram-positive bacteria. The two laser confocal microscope used in this study did not have the breadth of amplification wavelengths required to stimulate all three fluorescent dyes. Scanning electron microscopy pictures could also be done at later and earlier time points (the later points were not done as part of this study as the SEM broke while being used by someone else).

This chapter has shown that while there are differences in the structure of the different biofilms and as the biofilm ages, there are no obviously significant features of the three dimensional structure of the biofilm that would necessitate including a spatial component to the biofilm modelling done in Chapter 6 as the structures are surprisingly homogenous.

5 Transcriptome Analysis of *S. mutans*: Investigating the Effects of *V. dispar* Co-culture

5.1 Introduction

The previous chapters have outlined how growing two species together affects their population size, vitality, morphology and the effect they have on the pH of the environment over time. This chapter moves from the population level to the molecular level and reports upon how Microarray analysis was used to identify transcriptional changes occurring in *S. mutans* resulting from the co-culture of *V. dispar* and how gene expression changed in the mature biofilm.

Luppens et al. (2008) investigated the effect of *V. parvula* on gene expression of *S. mutans* grown in a dual-species biofilm in buffered broth in culture plates at a single time point of 48 hours. This chapter describes the development of this previous work (although this work was commenced without knowledge of this earlier work as it was not then published) where the effect of another bacterial species (*V. dispar*) on the global transcriptome of a target species (*S. mutans*) was investigated at multiple stages of biofilm development (maturation I [24 hours growth] and maturation II [72 hours growth]). As such, it is the only known whole genome investigation of co-culture at multiple stages of biofilm growth. *V. dispar* and *V. parvula* are closely related species that are both found in close proximity to *S. mutans* in the mouth and the differences the two species have on *S. mutans* could be negligible or significant but there is no reason in the literature to suggest their interactions with *S. mutans* would differ significantly.

There are four main approaches which can be done in conjunction when conducting a whole genome transcriptomic study: investigate individual genes that show differences in expression, investigate genes of known function that are hypothesized to be important to see whether their expression changes or not, investigate what is occurring in gene networks/families/COGs, and investigate patterns of expression of genes in spatial proximity in the genome. All of these approaches were employed in this study.

Table 5.A A breakdown of the *S. mutans* genome based upon gene function.

(Modified from http://www.ncbi.nlm.nih.gov/sutils/coxik.cgi?cut=45&gi=263)

COGs # proteins	Description	in genome (%)	in genus (%)	in Bacilli (%)	in Bacteria (%)
	Information storage and processing				
145	Translation	6.6453	7.0097	5.0451	4.2968
0	RNA processing and modification	0.0000	0.0000	0.0011	0.0148
174	Transcription	7.9743	6.7067	7.0115	5.9643
138	Replication, recombination and repair	6.3245	6.8270	5.1288	4.8261
	Cellular processes				
0	Chromatin structure and dynamics	0.0000	0.0000	0.0148	0.0269
19	Cell cycle control, mitosis and meiosis	0.8708	0.9512	0.8397	0.7650
69	Defence mechanisms	3.1622	2.4906	1.9380	1.2590
70	Signal transduction mechanisms	3.2081	2.9868	3.2774	3.9952
84	Cell wall/membrane biogenesis	3.8497	4.1148	3.9362	4.3931
7	Cell motility	0.3208	0.4491	0.7314	1.4575
0	Cytoskeleton	0.0000	0.0012	0.0058	0.0116
0	Extracellular structures	0.0000	0.0000	0.0037	0.0154
23	Intracellular trafficking and secretion	1.0541	1.3225	1.0525	1.7757
58	Posttranslational modification, protein turnover, chaperones	2.6581	2.7016	2.3431	2.9290
	Metabolism				
71	Energy production and conversion	3.2539	2.6332	3.4146	4.8217
147	Carbohydrate transport and metabolism	6.7369	7.4364	6.2083	4.9545
182	Amino acid transport and metabolism	8.3410	6.7798	7.5583	7.2726
66	Nucleotide transport and metabolism	3.0247	3.2155	2.4411	1.7769
48	Coenzyme transport and metabolism	2.1998	2.1582	2.6098	2.9700
45	Lipid transport and metabolism	2.0623	2.0462	2.2032	2.8606
79	Inorganic ion transport and metabolism	3.6205	4.0453	4.7357	4.5898
29	Secondary metabolites biosynthesis, transport, catabolism	1.3291	0.9665	1.4888	2.2675
	Poorly Characterised				
240	General function prediction only	10.9991	9.7678	10.9104	10.4609
154	Function unknown	7.0577	7.0085	7.1506	6.1400
334	Not in COGs	15.3071	18.3817	19.9500	20.1551

S. mutans has more of its genome devoted to defence mechanisms than bacteria do in general (see Table 5.A). This is understandable given the complex and species-rich community in which *S. mutans* lives. It has a very small proportion of its genome comprising genes that show similarity to motility genes, which again is unsurprising given that it is non-motile. It has a large proportion of its genome devoted to the transport and metabolism of carbohydrates, reflecting the habitat in which it lives and that it has evolved to use carbohydrates as its primary energy source. It also has a relatively large proportion of its genome devoted to nucleotide transport and metabo-

lism. This is discussed later with reference to competence being used as a nutritional strategy in addition to its function to acquire new genes.

The importance of lactic acid for these two species makes the gene that codes for lactate dehydrogenase which converts pyruvate into lactic acid of prime importance. Also the universal bacterial communication molecule produced by the LuxS enzyme, autoinducer-2 (AI-2) is of interest because of its role in communication within and between species. *S. mutans* produces autoinducer-2 and can detect it, whereas *V. dispar* can not produce it but it may recognise it as a signalling molecule. Furthermore, autoinducer-2 is also known to be important in acid resistance (Wen and Burne 2004). Many genes have been shown to be important for biofilm formation (see Table 1 of Shemesh et al. 2007) and these were investigated in this study. *S. mutans* is able to survive in highly acidic environments by transporting protons out of its cytoplasm and thus maintaining a neutral internal cellular pH and it does this using an F_0F_1-type ATPase transporter (coded for by operon SMU.1527 to SMU.1534) and if there are differences in expression in the genes for this pump it would indicate differences in the production of protons. *S. mutans* depends on carbohydrates to generate ATP which they need to survive acid stress.

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is a high affinity/multi-substrate enzyme complex and is the major carbohydrate transport system in *S. mutans* and other oral streptococci. PTS is very important and relatively more so during acid stress so that carbohydrates can be metabolised to produce ATP (which can be used to remove protons from the cytoplasm using the ATPase transporter). PTS comprises two general proteins, enzyme I (EI) and the heat-stable phosphocarrier protein HPr which are coded for by the *ptsI* and *ptsH* genes respectively. These core proteins work in conjunction with sugar-specific permeases known as enzyme II complexes (EII) which phosphorylate and internalise many different sugars. In addition to their role in sugar uptake, PTS components influence many other cellular processes including biofilm formation, carbon catabolite repression, and virulence gene expression (reviewed in Abranches et al. 2006). In addition to the PTS, carbohydrates are also transported by ATP-binding cassette (ABC) transporters (Ajdić and Pham 2007). Fourteen PTS and four ABC transporters have been found in *S. mutans* UA159 (Ajdić et al. 2002). The mannose-PTS of *S. mutans* takes up mannose, glucose and the glucose analogue 2-deoxy-glucose and it appears that $EIIAB^{Man}$ (2

domains of the mannose-PTS) but not EII^{Glc} (the glucose-PTS) is important in the regulation of energy metabolism, and that $EIIAB^{Man}$ profoundly influences CCR, PTS activity, biofilm formation and genetic competence whereas EII^{Glc} does not (Abranches et al. 2006). Carbon catabolite repression is the down-regulation of genes that code for enzymes involved in the utilisation of a non-preferred energy source when a preferred energy source is available and it is controlled by the PTS enzyme HPr and the transcriptional regulator CcpA (Titgemeyer and Hillen 2002).

Genes involved in genetic competence (bringing DNA in to the cell) are important, as through them bacteria can gain valuable DNA that can be integrated into the genome and competence genes can also be considered as as a possible nutritional transporter. Recent studies have identified that competence genes are coupled with lysis genes and it has been postulated that this could be a strategy to acquire nutrients (Finkel and Kolter 2001, Spoering and Gilmore 2006, Lemos and Burne 2008). In support of this theory, Ajdić et al. (2002) found a relatively large proportion of the *S. mutans* genome coupled to nucleotide transport and metabolism.

All known two-component signal transduction systems (14) which regulate gene-expression by conveying external signals to transcriptional machinery (Ajdić et al. 2002, Biswas et al. 2008) are of interest. Examples of this are the nutritional alarmones (p)ppGpp which down regulates growth and shift metabolic activity to focus on storage and survival in response to exogenous signals (Lemos et al. 2007, Nascimento et al. 2008). The stress regulon is particularly important to consider because of the importance of *S. mutans'* ability to cope with the changing and demanding environment it lives in, and this regulon has also been shown to be strongly linked with biofilm formation (Lemos and Burne 2008).

Any differences in expression of these genes are important results but it is also important to consider these genes if they show no change as this indicates these genes function in the same way despite the presence of *V. dispar*. While these are all highlighted as genes of interest to see how they change when *S. mutans* is co-cultured with *V. dispar*; PTS, CcpA, the stress regulon, and quorum sensing systems are particularly important in understanding strategies *S. mutans* uses to establish, persist and cause disease (Abranches et al. 2008).

S. mutans is well studied, has its genome sequenced and has microarrays developed for it while *V. dispar* is less well studied. Consequently the focus of this chapter, by necessity (and due to its clinical importance), is on *S. mutans*, but in the future when the genome of a veillonellae is sequenced it would be very interesting to identify what RNA is being transcribed in a mirror experiment of this study. This research was conducted in an attempt to identify and unravel the interactions that occur between these two species. Bacteria provide a way to study interactions at the most basic level as all interactions are controlled chemically by genes, the proteins they produce and the metabolites these produce. Microarrays allow the identification of the genes that are being transcribed and can be used to determine how these two species interact.

5.2 Materials and Methods

The methods used to conduct the transcriptomic analysis are outlined in Section 2.5 while this section gives an overview and the rationale behind the methods used and describes the experimental design. Figure 5.A is a diagram showing the protocol of these experiments, starting with the growth of the biofilm, followed by RNA preservation and extraction. RNA was preserved and extracted using the guanidium thiocyanate method (Siebert and Chenchik 1993) which can be used to differentially lyse different membranes (Waddell and Butcher 2007) allowing for the enrichment of *S. mutans* RNA. Guanidium thiocyanate lyses the *V. dispar* membrane, but not the *S. mutans* membrane, thus releasing the contents of the *V. dispar* cells allowing the unlysed S. *mutans* cells to be pelleted by centrifugation so the lysed *V. dispar* which are in suspension are tipped off with the rest of the supernatant.

There are a number of ways to design a gene expression profiling experiment, with the main differences being the number of channels scanned (one or two), and if there are two channels scanned what samples are chosen to be competitively hybridised. A common depiction of microarray data shows grids of green, yellow and red dots representing the comparison of two samples, with yellow dots indicating there are equal amounts of each type of cDNA (made from the RNA) binding to that dot, while green or red dots indicate one or other of the samples has more cDNA (from RNA) binding to the dot (Cy3 fluoresces green while Cy5 fluoresces red). This method works well when comparing only two conditions, but when there are more than two conditions, many more microarrays need to be used to directly compare all samples or conditions need to be compared indirectly (for example if A and B are run together and B and C are run together then A can be compared with C indirectly as they have both been run with B) but this reduces the power of the comparison and increases the error of the experiment.

In this study a common reference design was used. One and three day single and dual species biofilms were tested as dual-channel microarrays with the other channel, the common reference, consisting of genomic *S. mutans* DNA. This allows for the comparison of multiple different microarray hybridisations (see Figure 5.B) as they are all standardised against a common reference and avoids the need to dye swap (a technique done when comparing two different treatments because of minor

differences in the binding affinity and fluorescence of the two dyes). Genomic DNA was used as the common reference as it is easily and cheaply extracted and as it is extremely consistent. Using this method values of expression obtained for each condition could be compared against all other conditions directly.

Single and dual species Biofilms grown on nitrocellulose filters on agar

Common reference (*S. mutans*) and negative control (*V. dispar*) grown planktonically

Filters added to GTC solution, preserving RNA and enriching for *S. mutans* (selectively preserving *S. mutans* RNA and disposing of *V. dispar* RNA)

DNA extracted

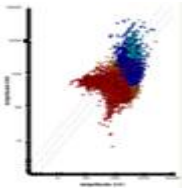
RNA extracted from enriched *S. mutans* pellet from previous step

PCR with random primers and dye labelling

Reverse transcription with random primers and dye labelling

Coupling hybridisation and slide washing

Scanning



Normalisation and analysis

Figure 5.A Simplified protocol of two channel microarray analysis.

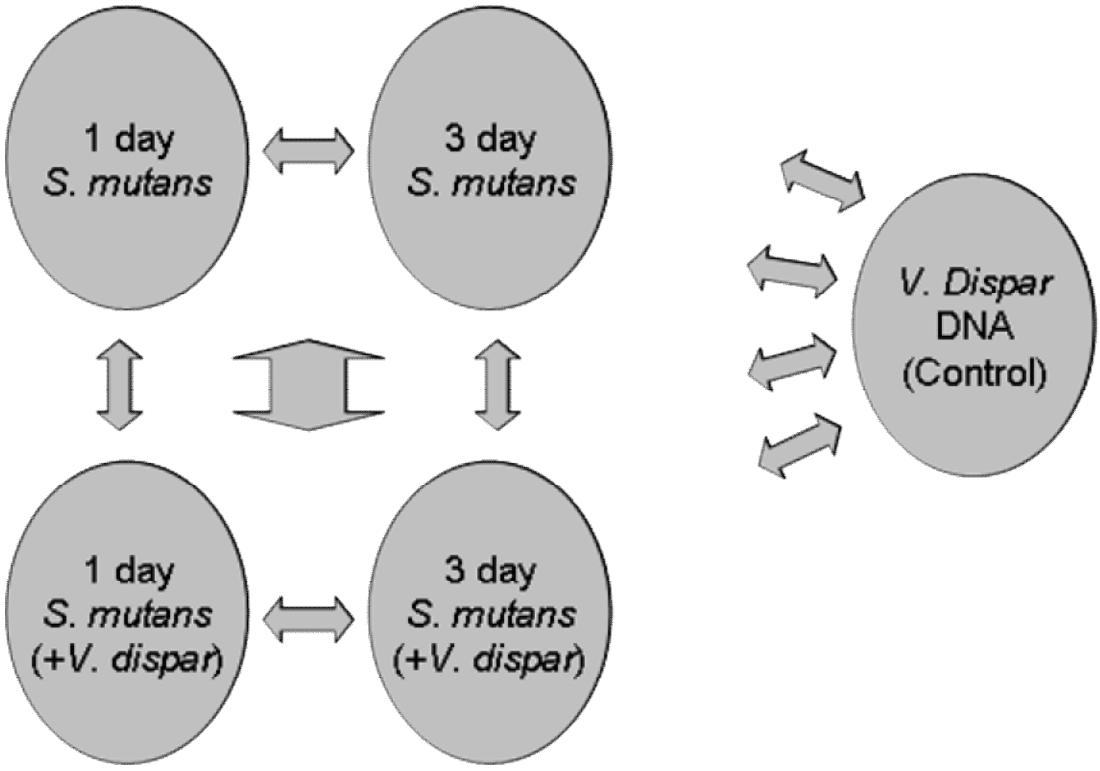


Figure 5.B Experimental design of the microarray experiments. There were four replicates of each of the conditions allowing comparisons to be made as represented by the arrows due to the common reference design of the experiment. Each replicate was a pool of 6 biofilms (6 filters with biofilms growing on them pooled together to extract sufficient RNA). In addition to hybridising *V. dispar* genomic DNA with the microarray as a negative control, negative controls were also integrated in to slides (500 *Arabidopsis thaliana* oligonucleotides).

Following hybridisation, slides were scanned with gain settings of between 65 and 70 (the max) in the Cy3 channel (green, 532nm stimulation, DNA) and between 55 and 70 in the Cy5 channel (red, 635nm stimulation, RNA). Quantification of the image array data was done using BlueFuse for Microarrays which applies a Bayesian approach to identify microarray spots and estimate their intensity taking in to account noise and background effects (BlueGnome, Cambridge, UK; Gottardo et al. 2005). Values were further normalised by performing the following normalisation procedures using GeneSpring; dye swap, per spot - divide by control channel, per gene - normalise to specific samples.

One-way ANOVA tests were done using GeneSpring using a p-value of 0.05 and Benjamini and Hochberg multiple testing correction (which uses a false discovery

rate rather than a family-wise error rate like Bonferroni correction) as it provides a good balance of detecting significant differences (avoiding Type II error) while protecting against false positives (Type I error, Benjamini and Hochberg 1995). This means that regardless of the number of different ANOVA performed, there is a global false positive error rate of 5% (an FWER, family wise error rate, of 5%). It is important to determine whether the genes differ statistically, but also whether the difference observed is biologically relevant. Many procedures have been developed to determine statistically whether genes differ in expression with the consensus that employing an FDR is good practice (Allison et al. 2006) but there is no benchmark for what constitutes biological relevance. This is as genes can differ by a few fold in expression with little change in phenotype, or can change very little and have a resulting change in phenotype. Luppens et al. (2008) who conducted a similar study used an FDR of 0.05 and a fold change of ≥ 1.3 for biological relevance.

In this study, to increase the power of the statistical test, only genes with a fold change of ≥ 1.5 were considered except for the comparison where single species (1 and 3 day combined) were compared against dual species (1 and 3 day combined) where no fold change was specified but the lowest fold change observed in the gene list produced, based upon the genes being determined to differ statistically significantly in expression, was a change of 1.3 fold (the same as the lower threshold in the Luppens et al. [2008] study). Dewing et al. (2003) investigating sexually dimorphic expression in mouse brains set the threshold arbitrarily low at 1.1 fold but using an FDR of 0.05 and a fold change threshold of 1.5 is common (e.g. Costigan et al. 2002, Summerfield and Sherman 2007, Raouf et al. 2008). The normalisations done using GeneSpring, but not BlueFuse, were taken off when calculating ANOVA. Results will be uploaded to the NIDCR website (following MIAME proposal [Brazma et al. 2001]).

5.3 Results

A variety of methods were trialled to extract RNA from the biofilms before identifying the enrichment method described in the Materials and Methods chapter (see Section 2.5). This method delivered the best quality and yield of RNA (determined using Nanodrop and Bioanalyser) in addition to enriching for *S. mutans* RNA over *V. dispar* RNA. Other methods did not enrich specifically for *S. mutans* RNA and delivered poorer quality RNA (Trizol; Bead beating, Trizol followed by bead beating).

This study identified many more genes, even when using a more stringent biological cut-off (1.5 fold difference as opposed to 1.3 fold), than a similar study done by Luppens et al. (2008) who identified 33 genes. Interestingly, there was no crossover in the genes they identified with the one day comparison reported in this study which identified 83 genes. Of the 599 genes identified in the three day comparison between single and dual species biofilms in this study there was a crossover of five genes with the Luppens et al. (2008) study. Two genes down-regulated in the three day comparison were the same as two up-regulated in Luppens et al. (2008) and three down-regulated were the same as three down-regulated, including the two most down-regulated genes in the Luppens et al. (2008) study (Cysteine aminopeptidase C *pepC* and a glucotransferase *malM*). Of the 65 genes identified in the comparison between *S. mutans* growing as a single species and as a dual species biofilm, there was a crossover of two genes which were down-regulated in both studies (Cysteine aminopeptidase C *pepC* which was the second most down-regulated gene in the Luppens et al. 2008 study and citrate lyase alpha chain *citF*).

5.3.1 Scanning the Microarrays

A representative scan is shown in Figure 5.C. The mostly black rectangle on the left shows where Cy3 and DNA fragments (the common reference, *S. mutans* DNA) have bound and fluoresced when stimulated with laser light (532nm). The scan is shown about twice the actual size of the microarray. The tiny white and grey dots in this rectangle are where the Cy3 was fluorescing. The top square in the middle shows an enlargement of part of this scan where the dots can be more easily seen. Each gene has four technical replicates in the array and in the enlargement this can be seen in

the four banks of nearly identical columns of dots. The similarity of the four banks of columns of dots shows how reproducible the data is. If there have been problems in the method they become immediately apparent if the patterns are not consistent. There are about 200 genes shown in this enlarged area with four replicates of each. On the right hand side of the figure is a scan of the same microarray slide, that has been hybridised to the same mix of molecules, but it shows the scan (stimulated with 635nm) of the Cy5 fluorescence, the RNA channel, and in the lower square it shows an enlargement of the same section of the array. The intensity differences of the different dots, caused by differing amounts of the different types of RNA are instantly apparent.

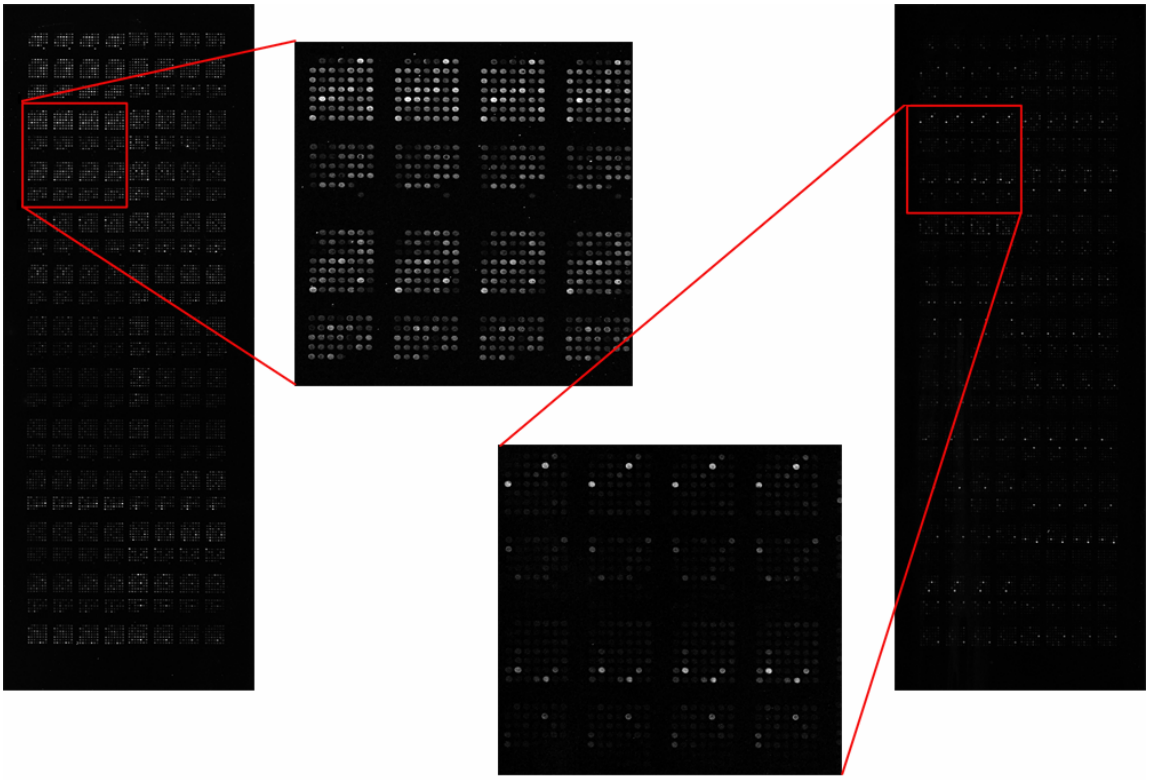


Figure 5.C Representative microarray data showing scans of Cy3 and Cy5 fluorescence. Scans showing fluorescence, left Cy3 (DNA) and right Cy5 (RNA), from a single species *S. mutans* biofilm, sample S1, with the two sections marked in red enlarged in the middle showing the spots where the fluorescent dyes bound to the oligonucleotides.

5.3.2 Normalised Gene Expression Results

The expression levels of all genes of the four replicates of the four conditions and of the negative control are shown in Figure 5.D. This figure shows all the normalized data of the experiments, and is a quick way to identify similarities and differences of the different conditions and to highlight any issues with the experiment. The dendrogram (tree) at the top of the figure shows how the four sets of four biological replicates all group together and that the control sits as an outlier. This figure indicates the extractions and experiment worked well but also shows the three day dual species scans are quite different to the earlier conditions. This is discussed later in the chapter. Also note how single species one day biofilms are more closely related (they are more closely connected by the branches of the tree) to dual species one day biofilms than single species three day biofilms. That is, the age of the biofilm and the resulting environment, are having a more powerful effect on *S. mutans* gene expression than co-culture with *V. dispar*.

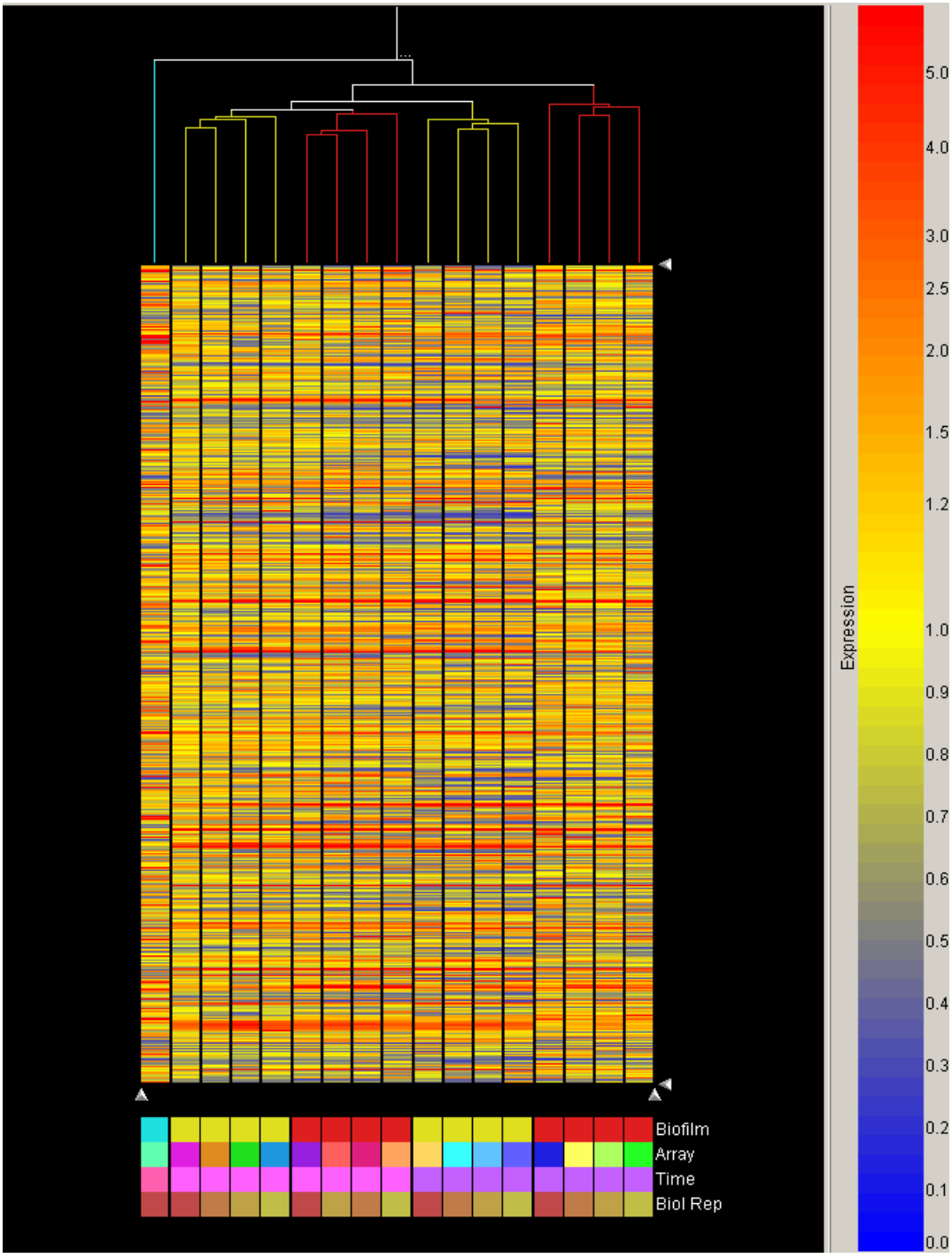


Figure 5.D Overview of normalized microarray data. Results of the seventeen microarrays scanned; four replicates of four conditions and the negative control. Microarray data experiment tree showing the seventeen microarrays scanned with the control column at the left, single species one day biofilms in columns two to five, dual species one day columns six to nine, single species three day in columns ten to thirteen and dual species three day biofilms in columns fourteen to seventeen. The linked lines at the top of the figure (in the shape of an upside down tree) indicate which microarray results were the most similar as the horizontal lines join the closest pairings.

5.3.3 Comparisons (Individual Gene Expression Differences and COGs)

Comparisons were made between single species and dual species biofilms at the one day time-point, three day time-point, with the time-points combined and between one-day single-species *S. mutans* biofilms and three-day single-species *S. mutans* biofilms.

5.3.3.1 Comparison of one day single and dual species biofilms

There are a number of comparisons and tests that can be made using the data, but the high yield and quality of the one day RNA extractions, and the direct comparison of single and dual species biofilms at an important stage of biofilm development (Maturation I) make the results in this section very important.

When gene expression levels of one day single species biofilm and one day dual species biofilms are compared (limiting the pool of tested genes to those with a 1.5 fold difference in expression or above to increase the power of the test), 83 genes are identified as being significantly differentially expressed (ANOVA, $p<0.05$, Benjamini and Hochberg multiple testing correction). These genes are shown in Figure 5.E; a graphical depiction of the changes in gene expression of the 83 significantly differentially expressed genes. The four replicates of each of the four conditions are shown alongside the negative control. The data have been normalized to the single-species one-day data, as evident by the tight clustering around the intensity of one (y-axis) for this condition. The differential expression is evident in the bifurcation of the gene expression intensities in the figure. There is no similarity in the pattern of expression for these genes in the three day biofilms. Each gene is represented by the connected lines (the negative control, one day biofilms and three day biofilms are not connected for clarity), and the lines are coloured by the relative amount of gene-expression of the fourth replicate of the dual species three day condition using a standard heat map colouration as shown on the right of the figure.

When comparing one day old single species *S. mutans* biofilms with dual species *S. mutans* and *V. dispar* biofilms, 56 genes were up-regulated more than 1.5 fold in dual species biofilms while 27 genes were down-regulated more than 1.5 fold in dual

species biofilms (ANOVA, Benjamini and Hochberg multiple testing correction, $p<0.05$). Of the 56 genes up-regulated, 29 of these are classed as hypothetical proteins, although 7 of these are assigned to COGs. However there are 22 hypothetical proteins of the 56 that are up-regulated that are classed as function unknown or are not in COGs. This large number of proteins with unknown function was tested to determine whether the observed number deviated from the number expected by chance. In the entire genome there are 488 genes that are classed as function unknown (154) or not in COGs (334) of the 1960 total genes (2182 if one sums the first column in Table 5.A as these genes were assigned using an automated system rather than the primary annotation that identified 1960 genes). Thus if a Chi-squared test is performed (calculating expected using 488/1960 rather than 488/2182 thus making the test more conservative), there are many more genes of unknown function (function unknown and not in COGs) that are up-regulated in dual species biofilms than would be expected by chance (Observed:22, Expected:13.9, χ^2=6.200, 1 d.f., two-tailed p=0.0128). However, there is no deviation from what would be expected by chance in the number of genes that are of unknown function (function unknown and not in COGs) that are down-regulated in dual species biofilms (Observed:3, Expected:6.72244898, χ^2=2.745, 1 d.f., two-tailed p=0.0976).

The actual genes that were shown to be differentially expressed are shown in Table 5.B (genes up-regulated in dual-species one day old biofilms) and in Table 5.C (genes down-regulated in dual-species one day old biofilms). The Tables show the loci, sorted by genome order, the function of the gene if it is known (definition), the gene name, observed fold-change in expression, the significance of this difference and the COG each individual locus belongs to. These Tables contain some very interesting genes including PTS genes, some of which are up-regulated while others are down-regulated, two endonucleases that are up-regulated in dual species biofilms, histidine kinases that are differentially expressed (two up-regulated, one down-regulated), the competence stimulating peptide is up-regulated in dual species biofilms, pyruvate dehydrogenase genes, and the chromosomal replication initiation protein is down-regulated in dual species one day old biofilms.

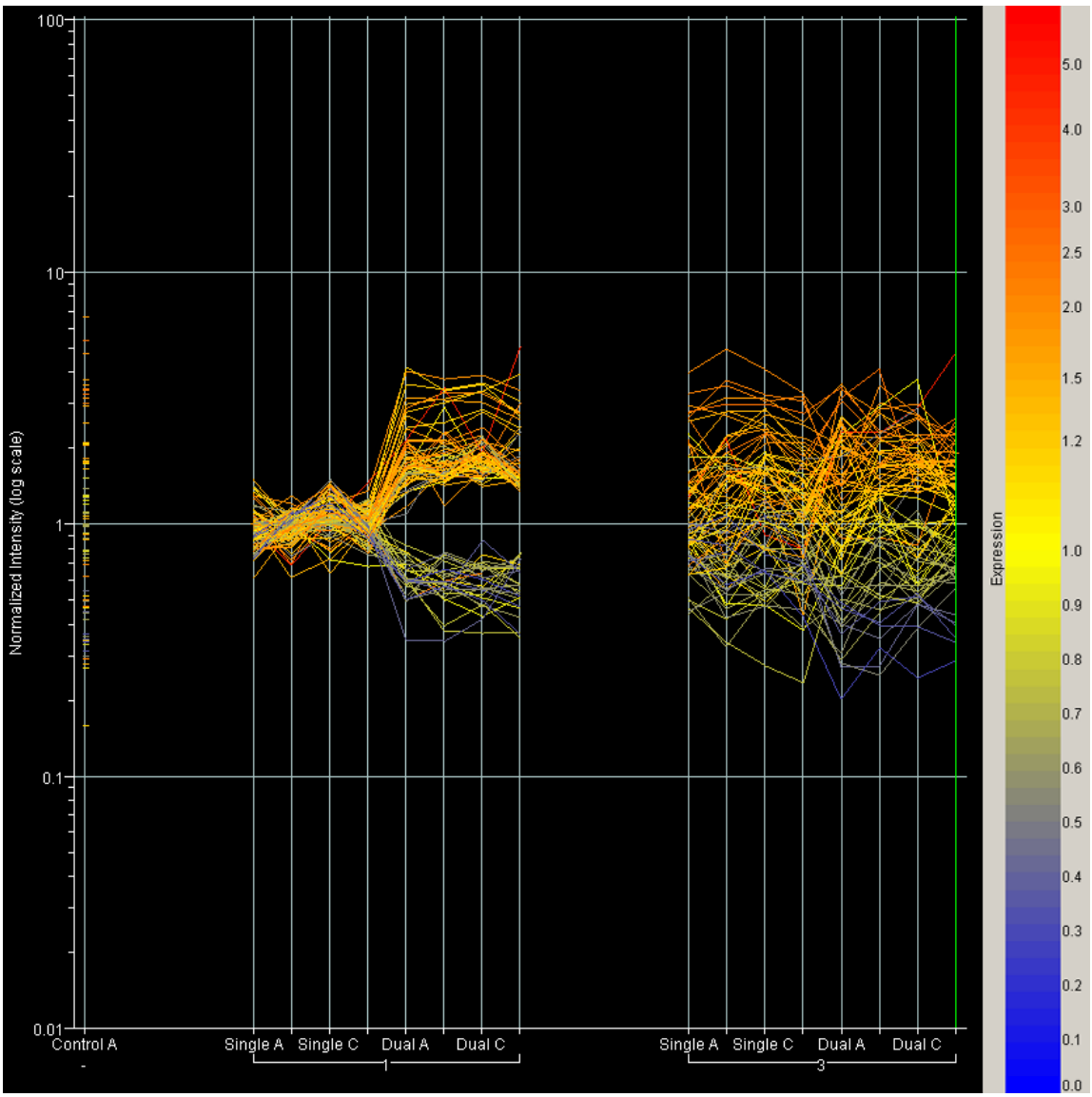


Figure 5.E Differential gene expression in one day old biofilms. Expression coloured by intensity of Dual 3 Day sample D (all samples are shown in the figure but the left side shows lines for 83 genes that differ in expression between single species and dual species biofilms while the right hand side shows how the same genes are expressed in single and dual species three day old biofilms).

[gene list: ANOVA P<0.05 B+H Single 1d vs. dual 1d 1.5-fold differences (83 genes)]

Table 5.B Genes up-regulated in dual-species one day old biofilms.

A fold change of 2.0 for a gene indicates that *S. mutans* growing in a dual-species biofilm transcribed on average twice as much mRNA from that gene as *S. mutans* growing as a single species biofilm (after one day of growth).

Locus	Definition	Gene name	Fold change	P-value	COG
SMU.18	hypothetical protein		1.8	0.0451	*Inorganic ion transport and metabolism*
SMU.104	putative alpha-glucosidase; glycosyl hydrolase		2.0	0.0356	*Carbohydrate transport and metabolism*
SMU.150	hypothetical protein		1.8	0.0208	*Not in COGs*
SMU.187c	hypothetical protein		1.7	0.0251	*Translation*
SMU.188c	Hsp33-like chaperonin	hslO	1.7	0.0445	*Posttranslational modification, protein turnover, chaperones*
SMU.194c	bacteriophage P2 associated		1.9	0.0175	*Not in COGs*
SMU.195c	hypothetical protein		1.8	0.0356	*Not in COGs*
SMU.252	hypothetical protein		1.9	0.0286	*Not in COGs*
SMU.360	glyceraldehyde-3-phosphate dehydrogenase	gapC	1.7	0.0356	*Carbohydrate transport and metabolism*
SMU.423	hypothetical protein		1.6	0.0208	*Not in COGs*
SMU.434	hypothetical protein		1.7	0.0451	*Not in COGs*
SMU.573	hypothetical protein		1.5	0.0324	*Carbohydrate transport and metabolism*
SMU.764	alkyl hydroperoxide reductase	ahpC	1.5	0.0451	*Posttranslational modification, protein turnover, chaperones*
SMU.768c	hypothetical protein		1.6	0.0451	*Not in COGs*
SMU.955	hypothetical protein		1.6	0.0124	*Function unknown*
SMU.956	putative Clp-like ATP-dependent protease, ATP-binding subunit	clp	1.6	0.0215	*Posttranslational modification, protein turnover, chaperones*
SMU.987	cell wall-associated protein precursor WapA	wapA	1.6	0.0124	*Cell wall/membrane biogenesis*
SMU.1095	putative choline ABC transporter, osmoprotectant binding protein	opuBc	1.5	0.0451	*Cell wall/membrane biogenesis*
SMU.1128	putative histidine kinase sensor CiaH	ciaH	1.6	0.0451	*Signal transduction mechanisms*
SMU.1192	DNA polymerase III DnaE	dnaE	1.6	0.0451	*Replication, recombination and repair*
SMU.1255c	hypothetical protein		2.0	0.0124	*Not in COGs*
SMU.1259	restriction endonuclease		2.9	0.0356	*Not in COGs*
SMU.1357	putative putative transposase		1.8	0.0216	*Not in COGs*
SMU.1389	hypothetical protein	pckA	1.6	0.0207	*Energy production and conversion*

SMU.1405c	hypothetical protein		1.9	0.0451	*Function unknown*
SMU.1419	putative transcriptional regulator		1.6	0.0208	*Transcription*
SMU.1420	putative oxidoreductase		1.7	0.0286	*General function prediction only*
SMU.1422	putative pyruvate dehydrogenase E1 component β subunit	pdhB	1.8	0.0445	*Energy production and conversion*
SMU.1423	putative pyruvate dehydrogenase, TPP-dependent E1 component α-subunit	pdhA	2.0	0.0215	*Energy production and conversion*
SMU.1424	putative dihydrolipoamide dehydrogenase	pdhD	1.8	0.0208	*Energy production and conversion*
SMU.1425	putative Clp proteinase, ATP-binding subunit ClpB	clpB	1.6	0.0251	*Posttranslational modification, protein turnover, chaperones*
SMU.1485c	putative endonuclease		1.5	0.0356	*Defence mechanisms*
SMU.1496	galactose-6-phosphate isomerase subunit LacA	lacA	1.6	0.0186	*Carbohydrate transport and metabolism*
SMU.1522	putative amino acid ABC transporter, integral membrane protein	glnP	1.6	0.0149	*Amino acid transport and metabolism*
SMU.1564	putative glycogen phosphorylase	glgP	1.5	0.0340	*Carbohydrate transport and metabolism*
SMU.1596	cellobiose phosphotransferase system IIC component	celD	1.5	0.0286	*Carbohydrate transport and metabolism*
SMU.1753c	hypothetical protein		1.7	0.0122	*Replication, recombination and repair*
SMU.1754c	hypothetical protein		1.9	0.0122	*Replication, recombination and repair*
SMU.1757c	hypothetical protein		1.6	0.0373	*Replication, recombination and repair*
SMU.1782	hypothetical protein		1.7	0.0413	*Not in COGs*
SMU.1903c	hypothetical protein		1.7	0.0451	*Not in COGs*
SMU.1904c	hypothetical protein		2.3	0.0127	*Not in COGs*
SMU.1905c	putative bacteriocin secretion protein		2.1	0.0175	*Not in COGs*
SMU.1906c	hypothetical protein		3.0	0.0009	*Not in COGs*
SMU.1907	hypothetical protein		2.5	0.0251	*Not in COGs*
SMU.1908c	hypothetical protein		3.7	0.0124	*Not in COGs*
SMU.1909c	hypothetical protein		3.5	0.0124	*Not in COGs*
SMU.1910c	hypothetical protein		3.3	0.0090	*Not in COGs*
SMU.1912c	hypothetical protein		3.0	0.0126	*Not in COGs*
SMU.1913c	putative immunity protein, BLpL-like		3.3	0.0090	*Not in COGs*
SMU.1914c	hypothetical protein		3.7	0.0160	*Not in COGs*
SMU.1915	competence stimulating peptide, precursor	comC	1.7	0.0451	*Not in COGs*
SMU.1916	putative histidine kinase of the competence regulon, ComD	comD	1.8	0.0175	*Signal transduction mechanisms*

SMU.1976c	hypothetical protein		1.9	0.0451	*Not in COGs*
SMU.2052c	hypothetical protein		1.8	0.0286	*Not in COGs*
SMU.2053c	hypothetical protein		1.7	0.0356	*Not in COGs*

Table 5.C Genes down-regulated in dual-species one day old biofilms.

A fold change of 2.0 for a gene indicates that *S. mutans* growing in a dual-species biofilm transcribed on average half as much mRNA from that gene as *S. mutans* growing as a single species biofilm (after one day of growth).

Locus	Definition	Gene name	Fold change	P-value	COG
SMU.01	chromosomal replication initiation protein	dnaA	1.8	0.0186	*Replication, recombination and repair*
SMU.114	putative PTS system, fructose-specific IIBC component		2.1	0.0204	*Carbohydrate transport and metabolism*
SMU.116	tagatose 1,6-diphosphate aldolase	lacD2	1.5	0.0413	*Carbohydrate transport and metabolism*
SMU.232	acetolactate synthase 3 regulatory subunit	ilvH	1.6	0.0456	*Amino acid transport and metabolism*
SMU.233	ketol-acid reductoisomerase	ilvC	1.7	0.0127	*Coenzyme transport and metabolism*
SMU.236c	putative transcriptional regulator		1.6	0.0399	*Transcription*
SMU.237c	putative integral membrane protein		2.4	0.0208	*Defence mechanisms*
SMU.438c	putative (R)-2-hydroxyglutaryl-CoA dehydratase activator-related protein		2.3	0.0380	*Function unknown*
SMU.486	putative histidine kinase		1.5	0.0186	*Signal transduction mechanisms*
SMU.533	anthranilate synthase component II	trpG	1.6	0.0356	*Coenzyme transport and metabolism*
SMU.875c	putative transposase, IS150-like		1.5	0.0456	*Replication, recombination and repair*
SMU.1007	putative ABC transporter, permease protein		1.6	0.0208	*Not in COGs*
SMU.1011	putative CitG protein	citG	1.7	0.0451	*Coenzyme transport and metabolism*
SMU.1070c	hypothetical protein		1.7	0.0356	*Signal transduction mechanisms*
SMU.1294	flavodoxin	flaW	1.5	0.0286	*Energy production and conversion*
SMU.1372c	hypothetical protein		1.7	0.0340	*Not in COGs*
SMU.1434c	putative glycosyltransferase		1.5	0.0124	*Cell wall/membrane biogenesis*
SMU.1472	putative single-strand DNA-specific exonuclease RecJ	recJ	2.0	0.0127	*Replication, recombination and repair*
SMU.1476c	putative GTP-binding protein		1.6	0.0124	*General function prediction only*
SMU.1601	6-phospho-beta-glucosidase	celA	1.6	0.0406	*Carbohydrate transport and metabolism*

SMU.1606	SsrA-binding protein	smpB	1.5	0.0286	*Posttranslational modification, protein turnover, chaperones*
SMU.1803c	hypothetical protein		1.6	0.0456	*Not in COGs*
SMU.1927	putative ABC transporter, ATP-binding protein		1.6	0.0451	*Defence mechanisms*
SMU.1946	hypothetical protein		1.7	0.0164	*Not in COGs*
SMU.1957	putative PTS system, mannose-specific IID component		2.0	0.0208	*Carbohydrate transport and metabolism*
SMU.1958c	putative PTS system, mannose-specific IIC component		1.8	0.0356	*Carbohydrate transport and metabolism*
SMU.1961c	putative PTS system, sugar-specific enzyme IIA component		2.0	0.0404	*Carbohydrate transport and metabolism*

Because of the number of genes involved, it is useful to look at the COGs these genes belong to (see Figure 5.F). The Replication, recombination and repair COG has four genes up-regulated and three genes down-regulated in dual-species one day biofilms. The clusters Posttranslational modification, protein turnover and chaperones and Energy production and conversion each have four genes up-regulated and one gene down-regulated in dual-species one day biofilms, and Coenzyme transport and metabolism has three genes down-regulated in dual species one day biofilms.

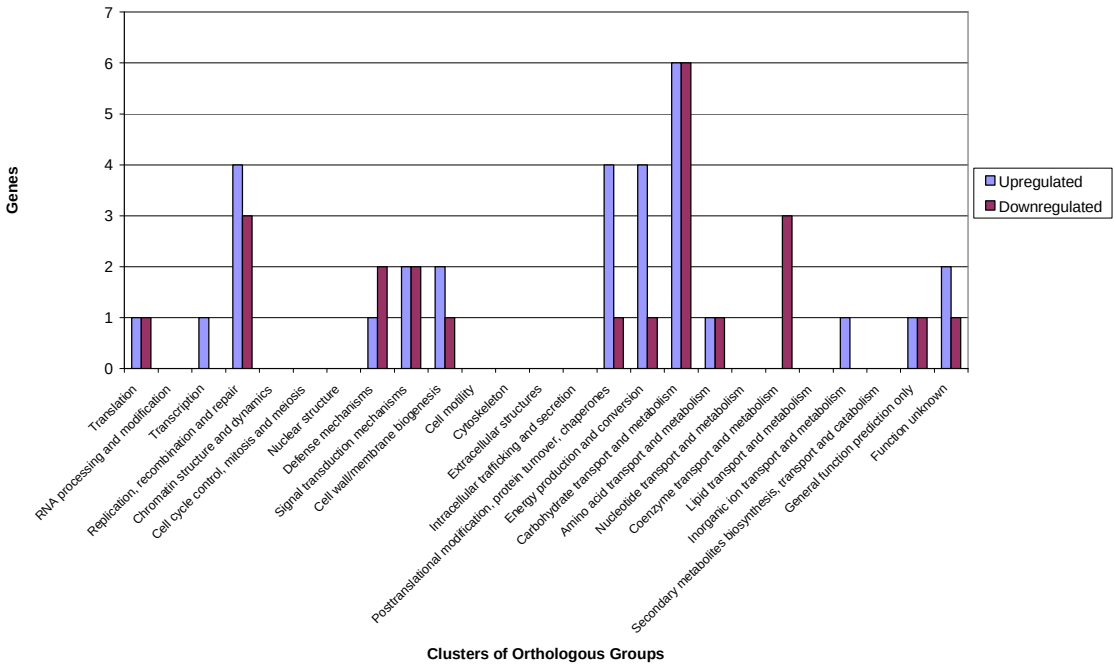


Figure 5.F Breakdown of differentially expressed genes between single and dual species one day old biofilms. A further 30 genes were not assigned to COGS: 26 genes up-regulated and 4 down-regulated.

[gene list: ANOVA P<0.05 B+H Single 1d vs. dual 1d 1.5-fold differences (83 genes)]

5.3.3.2 Comparison of three day single and dual species biofilms

599 genes were identified as being differentially expressed in the comparison of single and dual species biofilms that were three days old (ANOVA P<0.05, B+H, Single 3 day vs. dual 3 day 1.5-fold differences). However there was not much RNA in the dual species biofilms. This can be seen in the differences in intensity of expression shown in Figure 5.G. Figure 5.G shows boxplots of expression levels for raw data and for normalized data. This figure shows the lower intensity of the control (as expected) but also of the dual-species three day data (left half of the figure). The raw data is not totally raw as to quantify the scan it has been normalized against the signal of the immediate surroundings and across the four replicates.

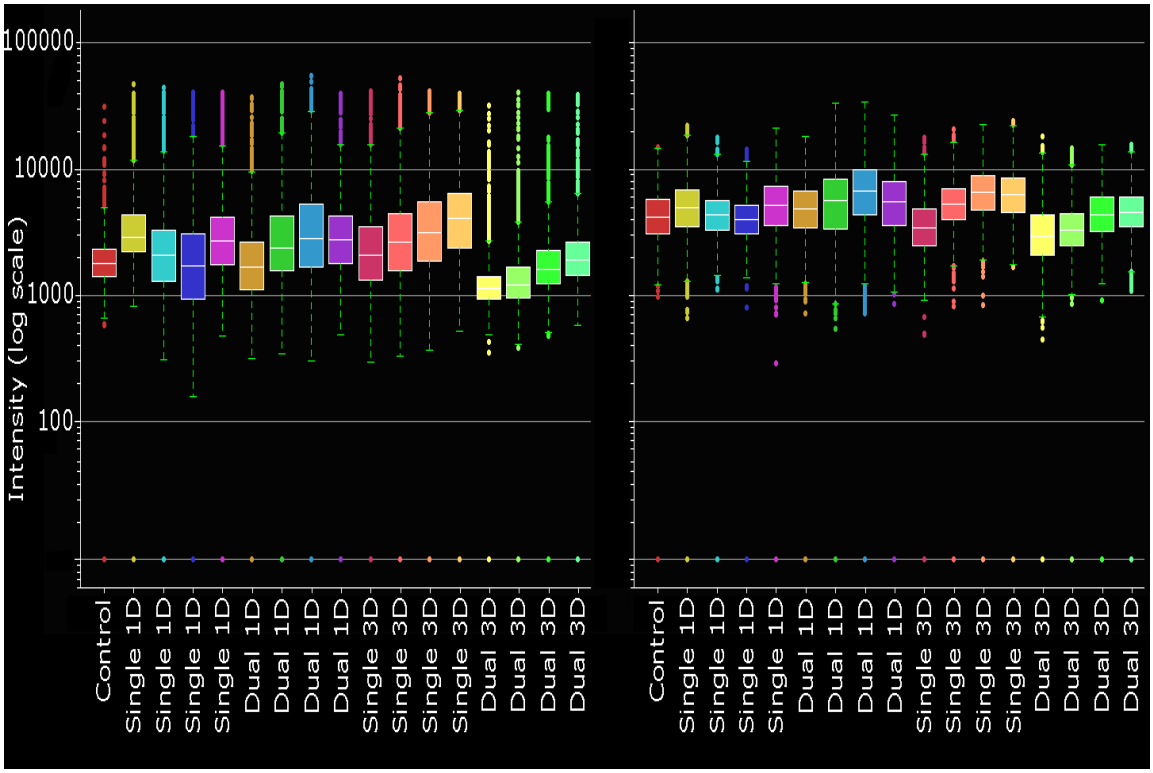


Figure 5.G Boxplots of gene expression levels. Minimally processed data is shown on the left and the normalized data is shown on the right. The y-axes show the 'Raw Intensity' plotted on a log scale. The x-axes show the control, then the four single-species one-day-old *S. mutans* biofilms, the four dual-species one-day-old biofilms, the four single-species three-day-old *S. mutans* biofilms and then the four dual-species three-day-old biofilms. The variability within and between samples is high.

The right half of the Figure shows the data after normalization with the intensity of the control and three day dual-species boxes pulled up.

The experiment worked in the three day dual species biofilms but provided low signal. Figure 5.H shows the Cy3 and Cy5 scans for SV1(3d), a representative 3 day dual-species biofilm scan and for the negative control. In the dual-species three day scan 18 sets (4 replicates) of spots are clearly visible in the scan while in the control 9 sets of spots are visible. The spots fluorescing in the negative control were excluded from the analyses. There was no crossover in the visible spots between the control and the three day dual species biofilms. In addition to normalization, the method of hybridisation can create some artefacts and an example of this is the horizontal stripe a quarter of the way up both scans. This is where the cover strips meet when doing the hybridization and there is slightly more solution which created more background signal. The normalization procedure subtracts background from each spot but chance events can lead to signal being processed. Equally nearly blank arrays can lead to chance events becoming signal and then becoming overrepresented due to normalisation. Both the three day dual species biofilms and the negative control are nearly blank scans in the Cy5 channel. This may have led to the large number of genes being identified as significantly differentially expressed in the three day comparison (See Figure 5.I).

Over 30% of the genome shows significant differences in expression. However there is a problem as the control channel shows a similarity in colouring based upon expression to the three day channels. The control channels is an essentially blank array with small amounts of noise unrelated to gene expression but probably due to tiny differences in the production of the spots and the oligonucleotides in the arrays. Once the data is normalized these minor differences, which may be reproducible even though they are unrelated to expression, may be classified as significant. The three day dual species data was of lower quality than the other conditions (due to there being less RNA initially) and while it contains differences that are real and due to differences in expression it may contain artefacts due to differences in the production of the array that have been made to appear significant due to normalization.

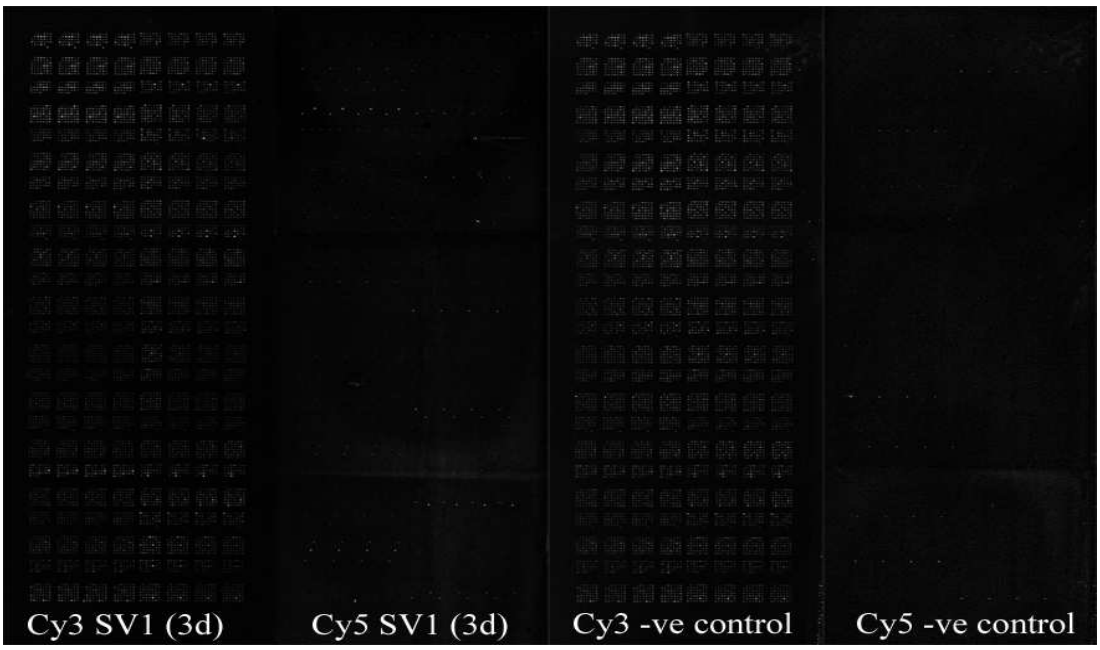


Figure 5.H Scans of a dual species 3 day microarray and the negative control. The two scans of a representative dual-species 3 day biofilm are shown on the left and the two scans of the negative control are shown on the right. Cy3 shows the DNA channel and Cy5 shows the test channel.

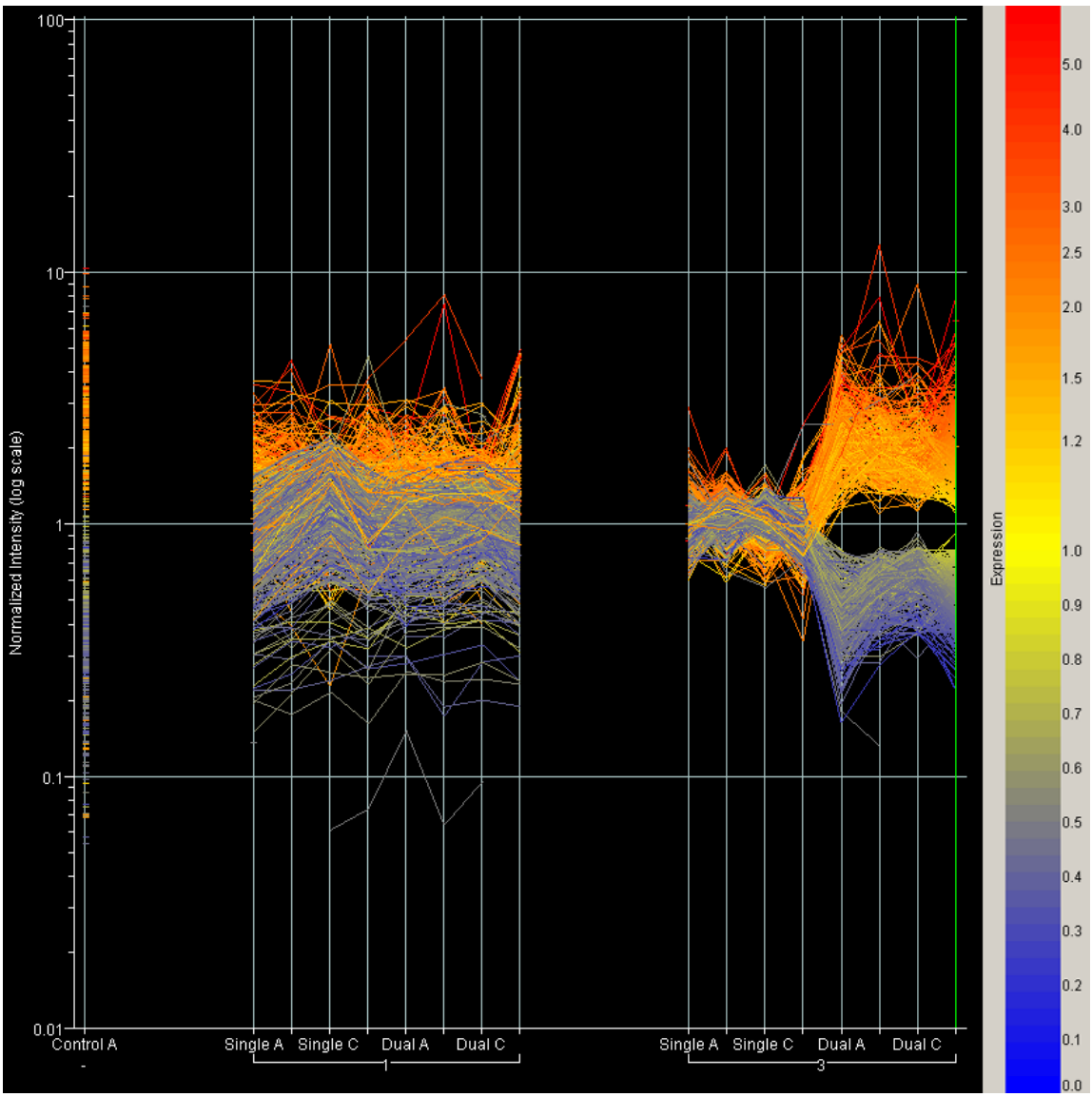


Figure 5.I Differential gene expression in three day old biofilms. Gene expression coloured by intensity of Dual 3 Day sample D.

[gene list: ANOVA P<0.05 B+H Single 3d vs. dual 3d 1.5-fold differences (599 genes)]

5.3.3.3 Comparison of single and dual species biofilms (both time points combined)

As the three day dual species data may have some error mixed in with the real signal a comparison was made that compares the combined time points for single species biofilms with the combined time points for dual species biofilms to allow the signal of the one day dual species time point to help exclude noise from the dual time point. In the combined time points, 65 genes were differentially expressed with 40 genes being up-regulated and 25 genes being down-regulated. All genes were compared rather than those showing a 1.5 fold change difference to identify more subtle

changes (although only genes with a 1.3 fold difference or above were found to be significantly differentially expressed). Of the 40 up-regulated genes 18 were the same as in the one day comparison, and of the 25 down-regulated genes six were the same as the one day comparison. The differentially expressed genes are shown in Figure 5.J and are listed in Table 5.D (up-regulated in dual species biofilms) and Table 5.E (down-regulated in dual species biofilms). The Tables are laid out as before but with the addition of an * if that gene was also significantly differentially expressed in the one day comparison.

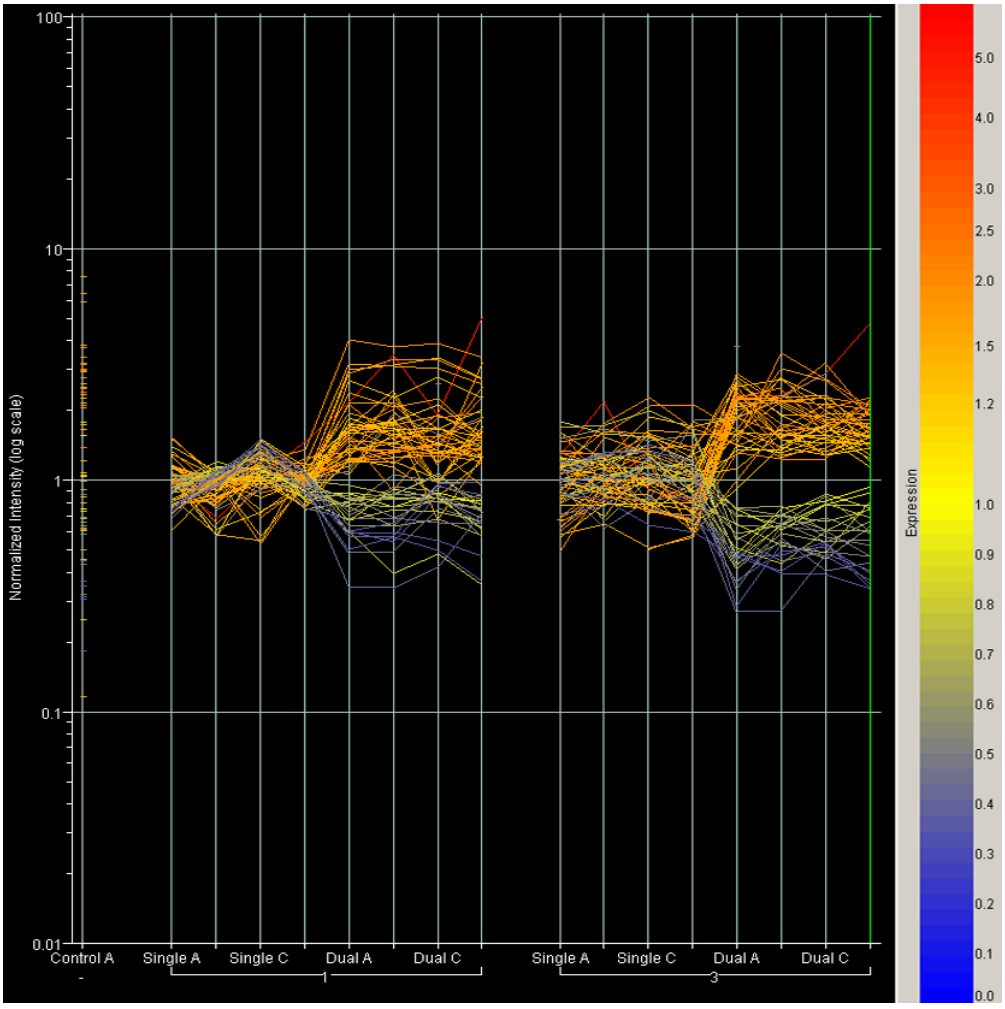


Figure 5.J Differential gene expression between single and dual species biofilms. Expression coloured by intensity of Dual 3 Day sample D.
[gene list: ANOVA P<0.05 B+H Single 1d+3d vs. dual 1d+3d (65 genes)]

Table 5.D Genes up-regulated in dual species biofilms compared with single species biofilms

A fold change of 2.0 for a gene indicates that *S. mutans* growing in a dual-species biofilm transcribed on average twice as much mRNA from that gene as *S. mutans* growing as a single species biofilm (after one and three days of growth combined).

Locus	Definition	Gene name	Fold change	P-value	COG	In s1v1
SMU.16	putative amino acid permease		1.8	0.0066	*Amino acid transport and metabolism*	
SMU.18	hypothetical protein		1.9	0.0066	*Inorganic ion transport and metabolism*	*
SMU.20	putative cell shape-determining protein MreC	mreC	2.1	0.0341	*Cell wall/membrane biogenesis*	
SMU.108	hypothetical protein		2.1	0.0486	*Translation*	
SMU.150	hypothetical protein		1.8	0.0138	*Not in COGs*	*
SMU.152	hypothetical protein		1.6	0.0342	*Not in COGs*	
SMU.187c	hypothetical protein		1.5	0.0478	*Translation*	*
SMU.188c	Hsp33-like chaperonin	hslO	1.7	0.0210	*Posttranslational modification, protein turnover, chaperones*	*
SMU.423	hypothetical protein		1.6	0.0142	*Not in COGs*	*
SMU.440	hypothetical protein		1.3	0.0308	*Not in COGs*	
SMU.478	guanylate kinase	gmk	1.3	0.0342	*Nucleotide transport and metabolism*	
SMU.485	hypothetical protein		1.4	0.0417	*Function unknown*	*
SMU.626	putative competence protein		1.7	0.0390	*General function prediction only*	
SMU.702c	putative transcriptional regulator		1.6	0.0488	*Function unknown*	*
SMU.771c	hypothetical protein		1.6	0.0488	*Not in COGs*	
SMU.836	hypothetical protein		1.8	0.0365	*General function prediction only*	
SMU.891	type I restriction-modification system DNA methylase	hsdM	1.7	0.0371	*Defence mechanisms*	
SMU.922	putative ABC transporter, ATP-binding protein		1.6	0.0488	*Defence mechanisms*	
SMU.980	putative PTS system, beta-glucoside-	bglP	1.6	0.0346	*Carbohydrate transport and metabolism*	

	specific EII component					
SMU.1128	putative histidine kinase sensor CiaH	ciaH	1.6	0.0139	*Signal transduction mechanisms*	*
SMU.1259	restriction endonuclease		2.7	0.0176	*Not in COGs*	*
SMU.1317c	hypothetical protein		3.2	0.0365	*Not in COGs*	*
SMU.1447c	hypothetical protein		1.5	0.0417	*General function prediction only*	
SMU.1491	PTS system, lactose-specific enzyme IIBC EIIBC-LAC)	lacE	1.7	0.0464	*Carbohydrate transport and metabolism*	
SMU.1495	galactose-6-phosphate isomerase subunit LacB	lacB	1.8	0.0478	*Carbohydrate transport and metabolism*	
SMU.1498	lactose repressor	lacR	1.8	0.0464	*Carbohydrate transport and metabolism*	
SMU.1644c	hypothetical protein		1.9	0.0417	*Not in COGs*	
SMU.1782	hypothetical protein		2.0	0.0038	*Not in COGs*	*
SMU.1788c	putative bacterocin transport accessory protein, Bta		1.6	0.0342	*Posttranslational modification, protein turnover, chaperones*	
SMU.1827	putative biotin biosynthesis protein		1.4	0.0488	*General function prediction only*	
SMU.1899	putative ABC transporter, ATP-binding and permease protein (fragment)		1.8	0.0464	*Defence mechanisms*	
SMU.1904c	hypothetical protein		1.8	0.0342	*Not in COGs*	*
SMU.1905c	putative bacteriocin secretion protein		1.9	0.0138	*Not in COGs*	*
SMU.1906c	hypothetical protein		2.1	0.0346	*Not in COGs*	*
SMU.1908c	hypothetical protein		2.2	0.0447	*Not in COGs*	*
SMU.1912c	hypothetical protein		2.1	0.0142	*Not in COGs*	*
SMU.1914c	hypothetical protein		2.2	0.0397	*Not in COGs*	*
SMU.1915	competence stimulating peptide, precursor	comC	2.0	0.0417	*Not in COGs*	*
SMU.2080	hypothetical protein		1.6	0.0488	*Signal transduction mechanisms*	
SMU.2165	putative SpoJ		2.3	0.0365	*Transcription*	

Table 5.E Genes down-regulated in dual species biofilms compared with single species biofilms

A fold change of 2.0 for a gene indicates that *S. mutans* growing in a dual-species biofilm transcribed on average half as much mRNA from that gene as *S. mutans* growing as a single species biofilm (after one and three days of growth combined).

Locus	Definition	Gene name	Fold change	P-value	COG	In s1v1
SMU.114	putative PTS system, fructose-specific IIBC component		1.7	0.0342	*Carbohydrate transport and metabolism*	*
SMU.249	putative NifS protein homolo-gue, class-V aminotransferase	nifS	1.7	0.0486	*Amino acid transport and metabolism*	
SMU.358	30S ribosomal protein S7		1.6	0.0417	*Translation*	
SMU.359	elongation factor G		1.6	0.0371	*Translation*	
SMU.438c	putative (R)-2-hydroxyglutaryl-CoA dehydratase activator-related protein		2.6	0.0066	*Function unknown*	*
SMU.466	cysteine aminopeptidase C	pepC	1.6	0.0478	*Amino acid transport and metabolism*	
SMU.486	putative histidine kinase		1.6	0.0210	*Signal transduction mechanisms*	*
SMU.488	putative hydrolase		1.4	0.0417	*General function prediction only*	
SMU.494	fructose-6-phosphate aldolase		1.5	0.0486	*Carbohydrate transport and metabolism*	
SMU.568	putative amino acid ABC transporter, ATP-binding protein		1.4	0.0417	*Amino acid transport and metabolism*	
SMU.624	putative 1-acylglycerol-3-phosphate O-acyltransferase		1.7	0.0417	*Lipid transport and metabolism*	
SMU.1011	putative CitG protein	citG	1.8	0.0417	*Coenzyme transport and metabolism*	*
SMU.1012c	putative transcriptional regulator		1.4	0.0488	*Transcription*	
SMU.1017	putative oxaloacetate decarboxylase, sodium ion pump subunit	oadB	2.0	0.0420	*Energy production and conversion*	
SMU.1021	putative citrate lyase, alfa subunit	cilA	1.9	0.0420	*Energy production and conversion*	

SMU.1023	oxaloacetate decarboxylase	pycB	2.0	0.0139	*Energy production and conversion*	
SMU.1623c	hypothetical protein		1.6	0.0417	*Function unknown*	
SMU.1665	putative branched chain amino acid ABC transporter, ATP-binding protein	livF	1.3	0.0488	*Amino acid transport and metabolism*	
SMU.1709	putative potassium uptake protein TrkH	trkH	1.6	0.0210	*Inorganic ion transport and metabolism*	
SMU.1791c	hypothetical protein		1.5	0.0430	*General function prediction only*	
SMU.1799	nicotinic acid mononucleotide adenylyltrans-ferase	nadD	1.6	0.0488	*Coenzyme transport and metabolism*	
SMU.1843	sucrose-6-phosphate hydrolase	scrB	1.4	0.0488	*Carbohydrate transport and metabolism*	
SMU.1957	putative PTS system, man-nose-specific IID component		2.1	0.0038	*Carbohydrate transport and metabolism*	*
SMU.1958c	putative PTS system, man-nose-specific IIC component		1.7	0.0331	*Carbohydrate transport and metabolism*	*
SMU.2028	levansucrase precursor; beta-D-fructosyltrans-ferase	sacB	1.8	0.0210	*Carbohydrate transport and metabolism*	

Figure 5.K shows a breakdown, based upon gene function, of differentially expressed genes in the comparison of single and dual species biofilms. This figure shows a similar distribution of gene expression amongst the COGs as seen in the comparison of single and dual species one day old biofilms (Figure 5.F) but with a few notable differences. Replication, recombination and repair no longer displays any significant difference. Three defence mechanisms rather than one are now up-regulated. Energy production and conversion has shifted from four up-regulated and one down-regulated to none up-regulated and three down-regulated.

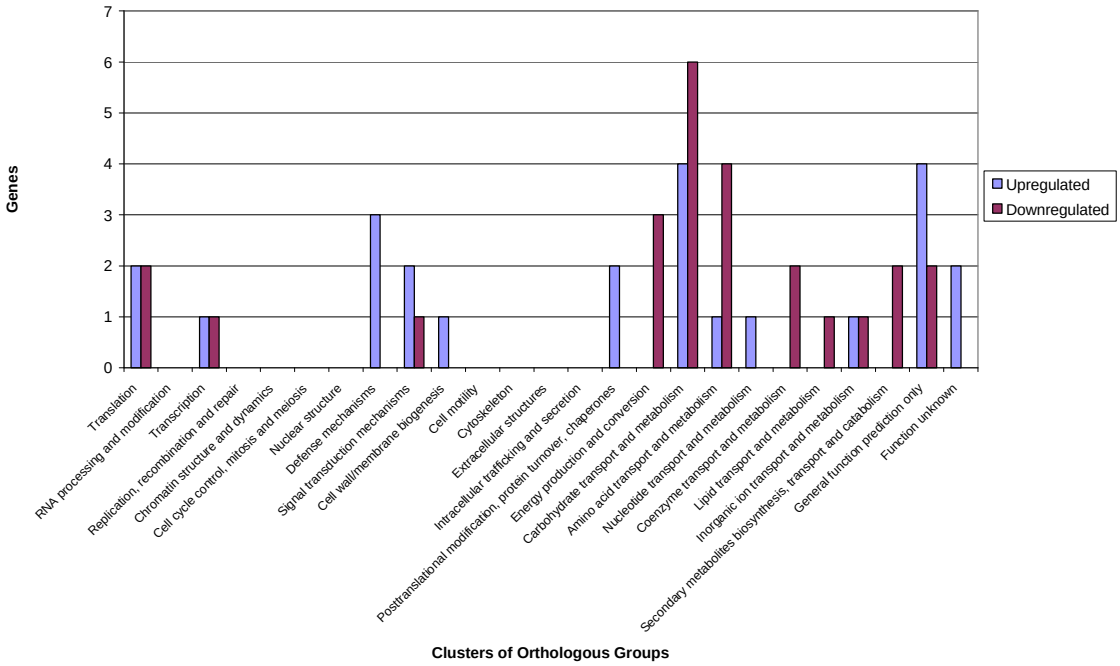


Figure 5.K Breakdown of differentially expressed genes between single and dual species biofilms. A further 16 genes were not assigned to COGS: 16 genes up-regulated and 0 down-regulated.
[gene list: ANOVA P<0.05 B+H Single 1d+3d vs. dual 1d+3d (65 genes)]

5.3.3.4 Comparison of one day *S. mutans* with 3 day *S. mutans* (single species biofilms)

92 genes were significantly differentially expressed in the three day single species biofilm when compared with the single species one day biofilm. Figure 5.L shows the genes that change in expression as the biofilm matures between one day and three days of growth (Maturation I and Maturation II). The conditions are arranged differently in this figure as they are paired by single or dual rather than by one day or three day.

The genes that were differentially expressed are shown in Table 5.F (up-regulated in three day single species biofilms) and in Table 5.G (down-regulated in three day single species biofilms).

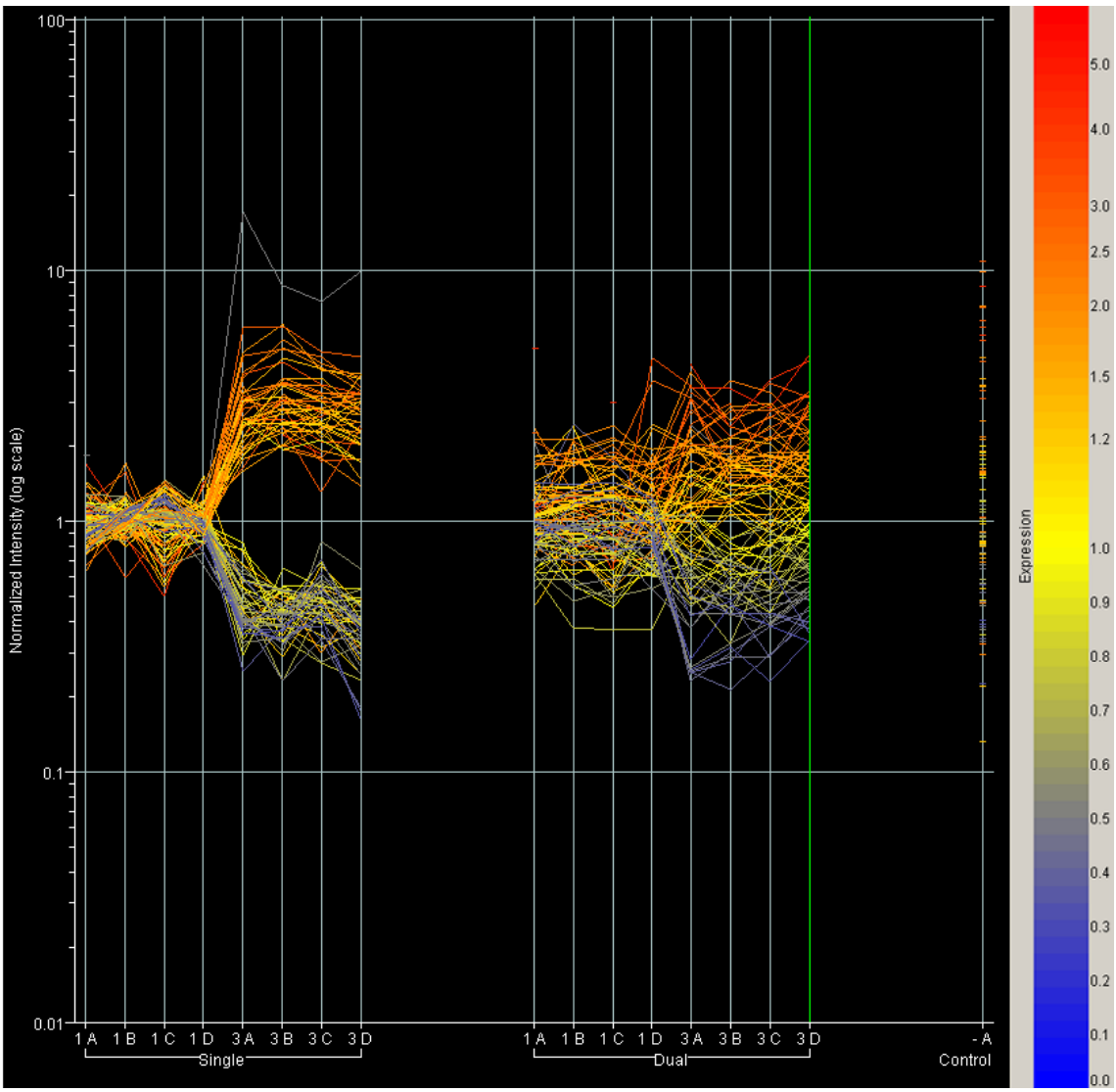


Figure 5.L Differential gene expression over time in single species *S. mutans* biofilms. Expression coloured by intensity of Dual 3 Day sample D.
[gene list: ANOVA P<0.05 B+H Single 1d vs. Single 3d (92)]

Table 5.F Genes up-regulated in three day single species *S. mutans* biofilms

A fold change of 2.0 for a gene indicates that *S. mutans* growing in three-days-old single-species biofilms transcribed on average twice as much mRNA from that particular gene as *S. mutans* growing in one-day-old single-species biofilms.

Locus	Definition	Gene name	Fold change	P-value	COG
SMU.21	putative cell shape-determining protein MreD	mreD	2.1	0.0106	*Not in COGs*
SMU.125	hypothetical protein		2.0	0.0011	*General function prediction only*
SMU.127	putative acetoin dehydrogenase (TPP-dependent), E1 component alpha subunit	adhA	2.9	0.0034	*Energy production and conversion*
SMU.128	putative acetoin dehydrogenase (TPP-dependent), E1 component beta subunit	adhB	4.4	0.0004	*Energy production and conversion*
SMU.129	branched-chain alpha-keto acid dehydrogenase subunit E2	adhC	5.3	0.0002	*Posttranslational modification, protein turnover, chaperones*
SMU.130	putative dihydroli-poamide dehydrogenase	adhD	2.8	0.0030	*Energy production and conversion*
SMU.131	putative lipoate-protein ligase	lplA	3.7	0.0007	*Coenzyme transport and metabolism*
SMU.132	putative hippurate amidohydrolase		2.7	0.0011	*General function prediction only*
SMU.191c	putative integrase		2.4	0.0485	*Replication, recom-bination and repair*
SMU.194c	bacteriophage P2 associated		2.2	0.0104	*Not in COGs*
SMU.195c	hypothetical protein		3.3	0.0008	*Not in COGs*
SMU.198c	putative conjugative transposon protein		2.7	0.0087	*General function prediction only*
SMU.200c	hypothetical protein		4.4	0.0011	*Not in COGs*
SMU.201c	putative transposon protein		3.2	0.0020	*Not in COGs*
SMU.206c	hypothetical protein		8.9	0.0042	*Not in COGs*
SMU.207c	putative transposon protein		3.3	0.0345	*Replication, recom-bination and repair*
SMU.209c	hypothetical protein		3.5	0.0012	*Not in COGs*
SMU.210c	hypothetical protein		2.3	0.0276	*Not in COGs*
SMU.211c	hypothetical protein		2.1	0.0095	*Not in COGs*
SMU.213c	hypothetical protein		3.2	0.0028	*Not in COGs*
SMU.215c	hypothetical protein		2.6	0.0024	*Not in COGs*
SMU.251	ABC transporter membrane protein		2.5	0.0007	*Posttranslational modification, protein turnover, chaperones*

SMU.252	hypothetical protein		2.1	0.0056	*Not in COGs*
SMU.764	alkyl hydroperoxide reductase	ahpC	2.5	0.0020	*Posttranslational modification, protein turnover, chaperones*
SMU.765	NADH oxidase/alkyl hydroperoxidase reductase peroxide-forming		2.1	0.0054	*Posttranslational modification, protein turnover, chaperones*
SMU.849	50S ribosomal protein L27	rpmA	2.0	0.0068	*Translation*
SMU.929c	hypothetical protein		2.5	0.0012	*Function unknown*
SMU.940c	putative hemolysin III		2.9	0.0007	*General function prediction only*
SMU.1316c	hypothetical protein		2.1	0.0446	*Not in COGs*
SMU.1116c	hypothetical protein		2.1	0.0446	*Not in COGs*
SMU.1418	coproporphyrinogen III oxidase	hemN	2.0	0.0028	*Coenzyme transport and metabolism*
SMU.1421	branched-chain alpha-keto acid dehydrogenase subunit E2	pdhC	4.3	0.0023	*Energy production and conversion*
SMU.1422	putative pyruvate dehydrogenase E1 component beta subunit)	pdhB	3.1	0.0030	*Energy production and conversion*
SMU.1423	putative pyruvate dehydrogenase, TPP-dependent E1 component alpha-subunit	pdhA	3.9	0.0011	*Energy production and conversion*
SMU.1424	putative dihydroli-poamide dehydrogenase	pdhD	3.4	0.0015	*Energy production and conversion*
SMU.1451	putative alpha-acetolactate decar-boxylase	aldB	2.7	0.0017	*Secondary metabo-lites biosynthesis, transport and catabolism*
SMU.1514	ribonuclease III	rnc	2.2	0.0417	*Transcription*
SMU.1561	putative potassium uptake system protein TrkB	trkB	2.1	0.0068	*Inorganic ion transport and metabolism*
SMU.1564	putative glycogen phosphorylase	glgP	2.5	0.0007	*Carbohydrate transport and metabolism*
SMU.1754c	hypothetical protein		3.1	0.0009	*Replication, recom-bination and repair*
SMU.1895c	hypothetical protein		2.3	0.0007	*Not in COGs*
SMU.1896c	hypothetical protein		2.9	0.0007	*Not in COGs*
SMU.1978	putative acetate kinase	ackA	2.4	0.0095	*Energy production and conversion*

Table 5.G Genes down-regulated in three day single species *S. mutans* biofilms

A fold change of 2.0 for a gene indicates that *S. mutans* growing in three-days-old single-species biofilms transcribed on average half as much mRNA from that particular gene as *S. mutans* growing in one-day-old single-species biofilms.

Locus	Definition	Gene name	Fold change	P-value	COG
SMU.02	DNA polymerase III subunit beta	dnaN	2.3	0.0056	*Replication, recombination and repair*
SMU.05	hypothetical protein		2.1	0.0063	*Function unknown*
SMU.109	permease (efflux protein)		3.7	0.0056	*Not in COGs*
SMU.119	putative alcohol dehydrogenase class III	adh	2.1	0.0026	*Energy production and conversion*
SMU.137	malate dehydrogenase	mleS	2.2	0.0095	*Energy production and conversion*
SMU.237c	putative integral membrane protein		3.2	0.0052	*Defence mechanisms*
SMU.265	carbamate kinase	arcC	2.0	0.0085	*Amino acid transport and metabolism*
SMU.341	putative deoxyribonuclease		2.1	0.0056	*Replication, recombination and repair*
SMU.375	hypothetical protein		2.0	0.0067	*Not in COGs*
SMU.406c	hypothetical protein		2.3	0.0226	*General function prediction only*
SMU.508	hypothetical protein		2.8	0.0070	*General function prediction only*
SMU.509	hypothetical protein		2.0	0.0011	*Function unknown*
SMU.512c	hypothetical protein		3.1	0.0020	*General function prediction only*
SMU.534	anthranilate phosphoribosyltransferase	trpD	3.0	0.0107	*Amino acid transport and metabolism*
SMU.535	indole-3-glycerol-phosphate synthase	trpC	2.2	0.0053	*Amino acid transport and metabolism*
SMU.538	tryptophan synthase subunit alpha	trpA	2.6	0.0074	*Amino acid transport and metabolism*
SMU.658	hypothetical protein		2.0	0.0067	*Not in COGs*
SMU.660	putative histidine kinase SpaK		2.0	0.0331	*Signal transduction mechanisms*
SMU.664	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	argJ	2.6	0.0036	*Amino acid transport and metabolism*
SMU.666	acetylornithine aminotransferase	argD	2.3	0.0013	*Amino acid transport and metabolism*
SMU.671	citrate synthase	citZ	2.1	0.0009	*Energy production and conversion*
SMU.739c	hypothetical protein		2.3	0.0057	*Not in COGs*
SMU.946	putative permease		2.0	0.0036	*Coenzyme transport and metabolism*
SMU.1155	hypothetical protein		2.0	0.0012	*Not in COGs*

SMU.1209c	hypothetical protein		2.5	0.0131	*Not in COGs*
SMU.1268	imidazoleglycerol-phosphate dehydratase	hisB	2.6	0.0002	*Amino acid transport and metabolism*
SMU.1282	putative transcriptional regulator		2.8	0.0052	*Transcription*
SMU.1284c	hypothetical protein		2.3	0.0172	*General function prediction only*
SMU.1352	putative transposase		2.3	0.0020	*Replication, recombination and repair*
SMU.1353	putative transposase		3.0	0.0020	*Not in COGs*
SMU.1356c	putative putative transposase		2.0	0.0359	*Not in COGs*
SMU.1361c	TetR family transcriptional regulator		2.4	0.0125	*Transcription*
SMU.1369	hypothetical protein		2.6	0.0104	*Not in COGs*
SMU.1372c	hypothetical protein		2.3	0.0052	*Not in COGs*
SMU.1390	hypothetical protein		2.7	0.0012	*Not in COGs*
SMU.1399	hypothetical protein		2.0	0.0023	*Not in COGs*
SMU.1402c	hypothetical protein		2.5	0.0017	*Not in COGs*
SMU.1404c	hypothetical protein		2.1	0.0040	*Replication, recombination and repair*
SMU.1438c	putative Zn-dependent protease		2.1	0.0485	*Posttranslational modification, protein turnover, chaperones*
SMU.1675	cystathionine gamma-synthase	metB	3.4	0.0107	*Amino acid transport and metabolism*
SMU.1781	hypothetical protein		2.4	0.0031	*Translation*
SMU.1805	putative transcriptional regulator		2.2	0.0063	*Transcription*
SMU.1817c	putative maturase-related protein		2.4	0.0017	*Replication, recombination and repair*
SMU.1873	ribonuclease HIII	rnh3	2.1	0.0041	*Replication, recombination and repair*
SMU.1950	putative pseudouridylate synthase		2.2	0.0135	*Translation*
SMU.1966c	putative periplasmic sugar-binding protein		2.4	0.0045	*Carbohydrate transport and metabolism*
SMU.2005	adenylate kinase	adk	2.4	0.0054	*Nucleotide transport and metabolism*
SMU.2108c	putative transcriptional regulator		2.1	0.0135	*Transcription*
SMU.2109	putative MDR permease; multidrug efflux pump		2.5	0.0095	*Not in COGs*

Of note are the eight energy production and conversion genes (*adh** and *pdh**) that are up-regulated in the three day single species *S. mutans* biofilms (see Figure 5.M). These enzymes are at the tail end of the glycolytic pathway if pyruvate is not converted into lactic acid and the alternative path is taken. Also a number of hypothetical genes are up-regulated in three day old single species biofilms including

SMU.206c which has the highest fold-change of all the genes identified in this study (8.9 fold up-regulation) and there are many genes up-regulated in its vicinity. The best blast hit for SMU.206c is for 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase [*Photobacterium* sp. SKA34] but the e-value is only 3.8. SMU.191 to SMU.226 (which encompasses the up-regulated genes) has been identified as an open reading frame which includes many elements of a transposon (Region 5, Maruyama et al. 2009).

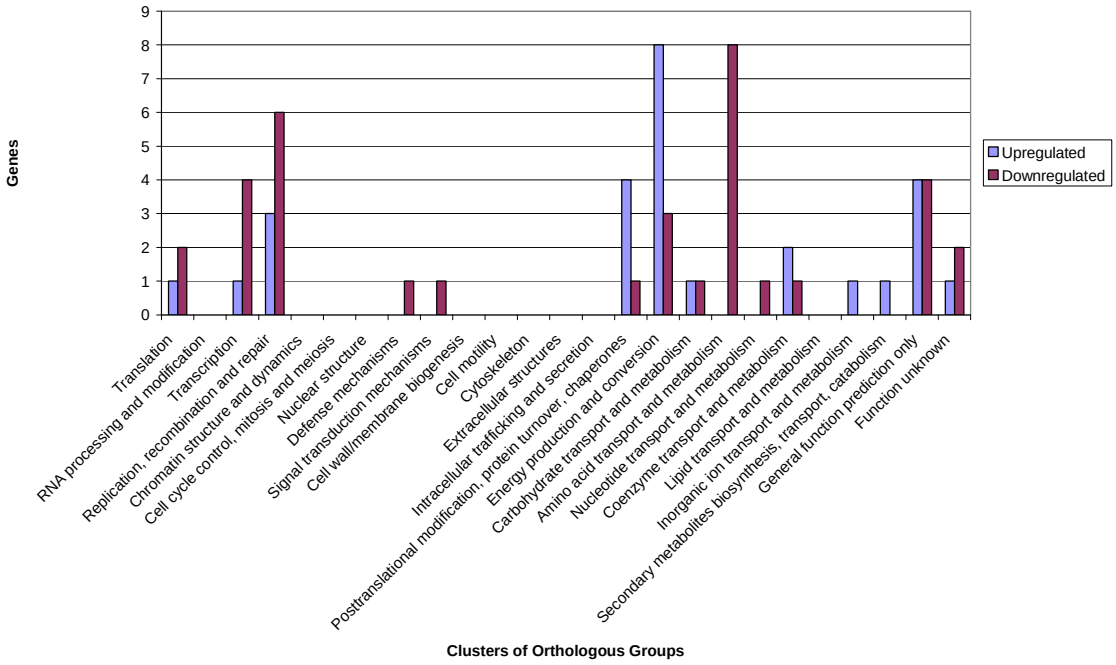


Figure 5.M Breakdown of differentially expressed genes between single species one day biofilms and single species three day biofilms.
[gene list: ANOVA P<0.05 B+H Single 1d vs. Single 3d 2-fold differences (92 genes)]

Note that an integrase and transposon were up-regulated (and also a transposon down-regulated). *adh* and *pdh* genes were up-regulated as well as glycogen phosphorylase, while amino acid synthases were down-regulated.

5.3.3.5 Comparison of one day *S. mutans* with 3 day *S. mutans* (dual species biofilms)

217 genes were identified as being differentially expressed in dual species biofilms as they mature but these are not investigated further as in Figure 5.N the

control channel shows similar expression levels of the differentially expressed genes due to poor signal in the three day dual species biofilms (as seen earlier in Figure 5.I).

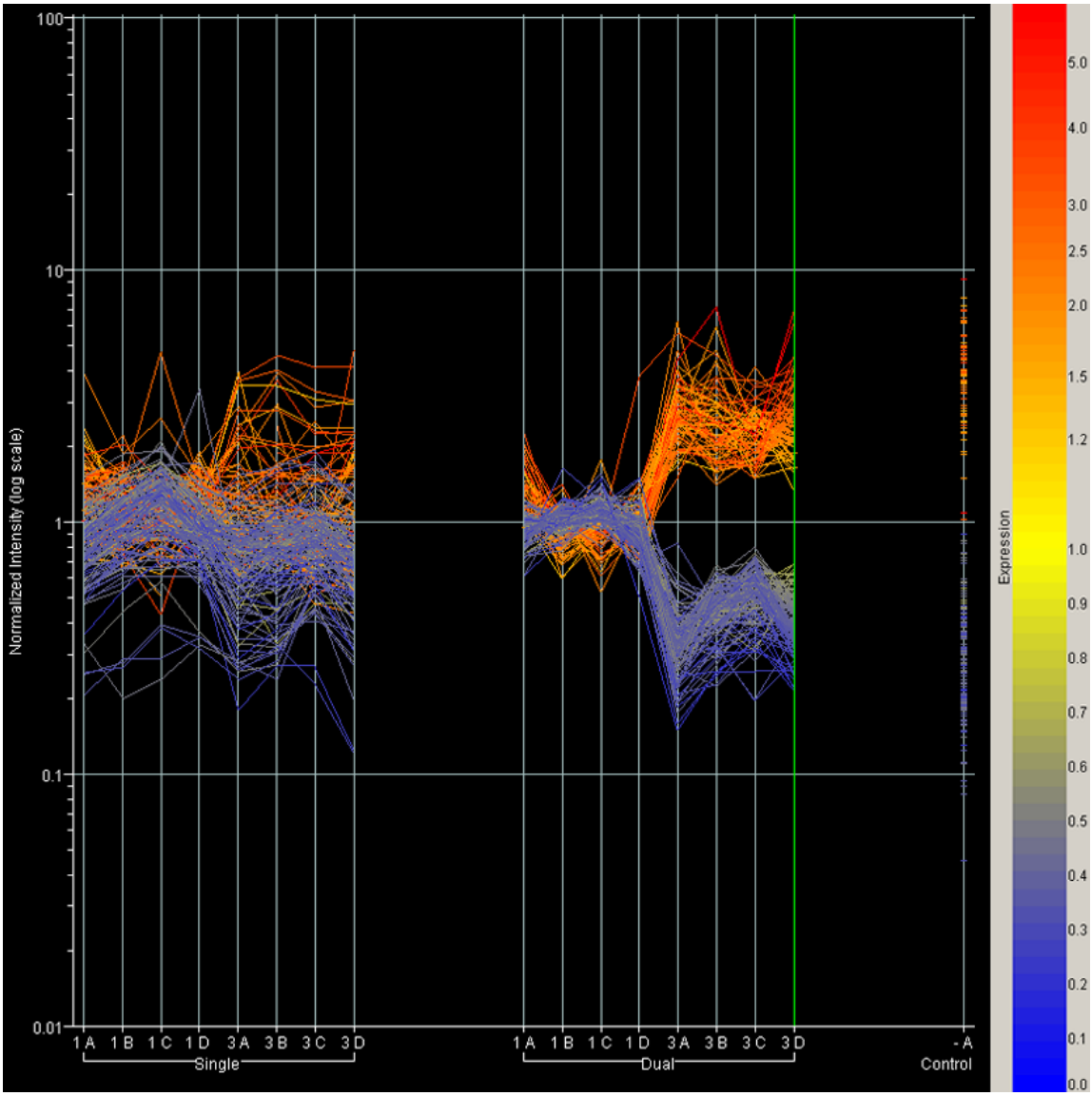


Figure 5.N Differential gene expression over time in dual species biofilms. Expression coloured by the intensity of three day dual species biofilms.
[gene list: ANOVA P<0.05 B+H Dual 1d vs. Dual 3d (217 genes)]

5.3.4 Spatial Analysis of Gene Expression Changes within the Genome

Loci were presented in genome order in Tables 5.B to 5.G as adjacent genes are often transcribed together. This section focuses on a few regions where adjacent genes were differentially expressed. Analysing the proximity of genes can identify potential mechanisms of control and may potentially identify genes that serve a

similar function or respond to similar conditions as they may come under the same regulatory control.

Table 5.H shows a bioinformatic investigation of genes that were upregulated in dual species biofilms (both one day and one and three day combined). In Table 5.H blue rectangles indicate membrane associated domains, pink rectangles indicate regions of low complexity, Bacteriocin IIc regions indicate bacteriocin motifs and the horizontal black line indicates the length of the protein. The third column, from BLAST and domain indicates putative functions for the different genes. SMU.1914c is classified as mutacin V, a bacteriocin, in a paper by Hale et al. (2005) but this has not been annotated to the genome sequence yet. These genes are shown in Figure 5.O alongside their surrounding genes and with putative functions labelled.

In Table 5.H Domains were analysed and visual representations were made using Mistdb.com (MiST2 Microbial Signal Transduction Database) and in Figures 5.O to 5.U repesentations of the chromosome were generated using Biocyc.org (BioCyc collection of pathway/genome databases) and subsequently annotated.

Table 5.H Putative classification of region coding for putative bacteriocin

Gene	Annotated function	From BLAST and domains	Domains
SMU.1903c	hypothetical protein	class II bacteriocin membrane transporter	
SMU.1904c	hypothetical protein	bacteriocin transport accessory protein	
SMU.1905c	putative bacteriocin secretion protein	bacteriocin secretion protein	
SMU.1906c	hypothetical protein	bacteriocin BlpU-like	
SMU.1907	hypothetical protein	hypothetical protein	
SMU.1908c	hypothetical protein	hypothetical protein (membrane associated)	
SMU.1909c	hypothetical protein	permease	
SMU.1910c	hypothetical protein	bacteriocin transporter/ histidine kinase	
SMU.1912c	hypothetical protein	hypothetical protein	
SMU.1913c	putative immunity protein, BLpL-like	immunity protein, BLpL-like	
SMU.1914c	hypothetical protein	mutacin V	

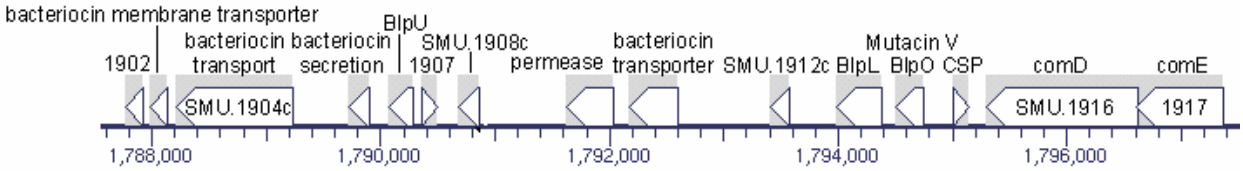


Figure 5.O Section of DNA showing genes SMU.1902 to SMU.1917 This region shows the putative bacteriocin region and surrounding genes. Putative functions (some determined in this study using the bioinformatic classifications made in Table 5.H) are shown for most of the genes that are classified as hypothetical proteins.

Figures 5P to 5.V show genes that are in close proximity that have been up- or down-regulated in the different comparisons. The following figures are in gene order and how they were differentially expressed is stated in the legend.

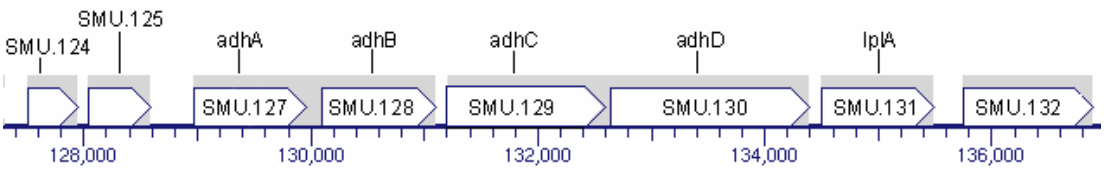


Figure 5.P Section of DNA showing genes SMU.124 to SMU.132 This region shows genes up- regulated in ageing *S. mutans* biofilms that are in close proximity to other up-regulated genes. Genes SMU.125, SMU.127, SMU.128, SMU.129, SMU.130, SMU.131 and SMU.132 which code for hypothetical proteins, *adhA, adhB, adhC, adhD, lplA,* and a putative hippurate amidohydrolase were up-regulated in ageing *S. mutans* biofilms.

The genes shown in Figure 5.P, which are upregulated in ageing *S. mutans* biofilms, code for enzymes that are at the tail end of the glcolytic pathway on the branch where pyruvate is not converted in to lactic acid.

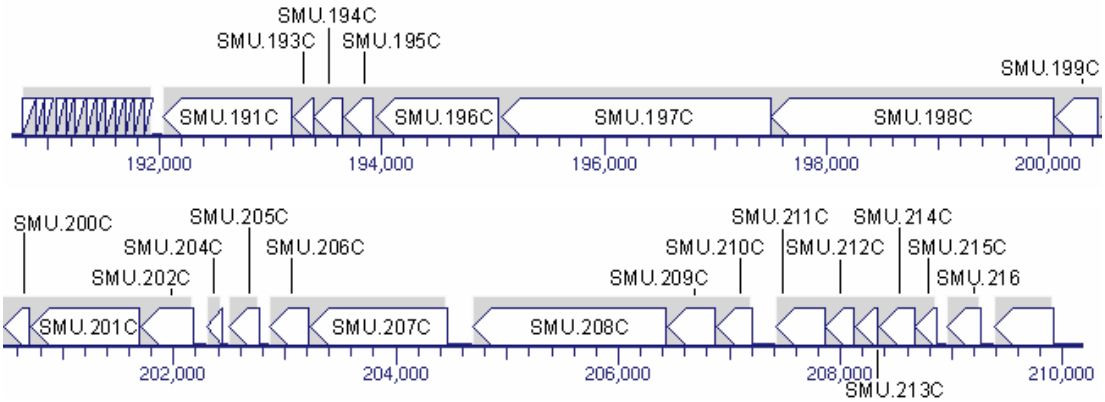


Figure 5.Q Section of DNA showing genes SMU.191C to SMU.216 This region shows genes up- regulated in ageing *S. mutans* biofilms that are in close proximity to other up-regulated genes. The genes SMU.191c, SMU.194c, SMU.195c, SMU.200c, SMU.201c, SMU.206c, SMU.207c, SMU.209c, SMU.210c, SMU.211c, SMU.213c and SMU.215c were all up-regulated and these genes include a putative integrase, a putative conjugative transposon protein, two putative transposon proteins, a bacteriophage P2 associated protein and many hypothetical proteins (SMU.191 to SMU.226 is open reading frame Region 5, Maruyama et al. 2009). The genes SMU.194c and SMU.195c were also up-regulated in dual species one day biofilms. These genes are not in COGs but one is bacteriophage P2 associated while the other is a hypothetical protein. The saw-tooth region at the left is a stretch of many small RNA genes.

Many genes were upregulated in dual-species one-day-old biofilms that were in close proximity, that are possibly all on a transposon that is being upregulated, and these are shown in Figure 5.Q.

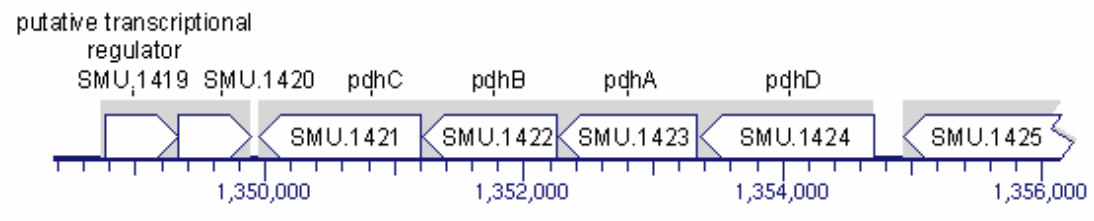


Figure 5.R Section of DNA showing genes SMU.1419 to SMU.1425 This region shows genes up- regulated in ageing *S. mutans* biofilms that are in close proximity to other up-regulated genes. SMU.1421, SMU.1422, SMU.1423 and SMU.1424 were all up-regulated and these genes code for *pdhA*, *pdhB*, *pdhC* and *pdhD* all parts of a putative pyruvate dehydrogenase. SMU.1422, SMU.1423 and SMU.1424 were also up-regulated in dual species one day biofilms.

As with the *adh* genes shown in Figure 5.P, which are upregulated in ageing *S. mutans* biofilms, the *pdh* genes shown in Figure 5.R are an alternative way to get energy from the breakdown of pyruvate without converting it to lactic acid.

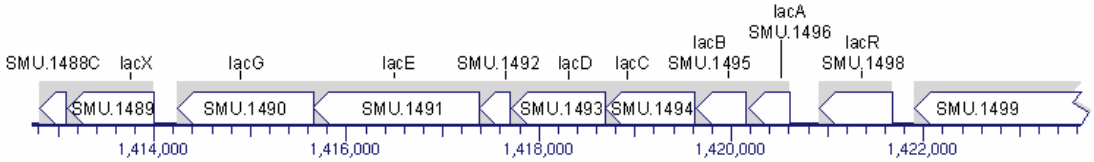


Figure 5.S Section of DNA showing genes SMU.1488C to SMU.1499 This region shows genes up-regulated in dual species (one and three days of growth combined) biofilms that are in close proximity to other up-regulated genes and the surrounding genes. SMU.1491, SMU.1495 and SMU.1498 (*lacE*, *lacB* and *lacR*) were all up-regulated in dual species biofilms.

Figure 5.S shows genes of the lactose operon. One gene from each of three components of this operon (the sugar-specific phosphotransferase system, tagatose 6-phosphate gene cluster and repressor) was upregulated in dual species biofilms.

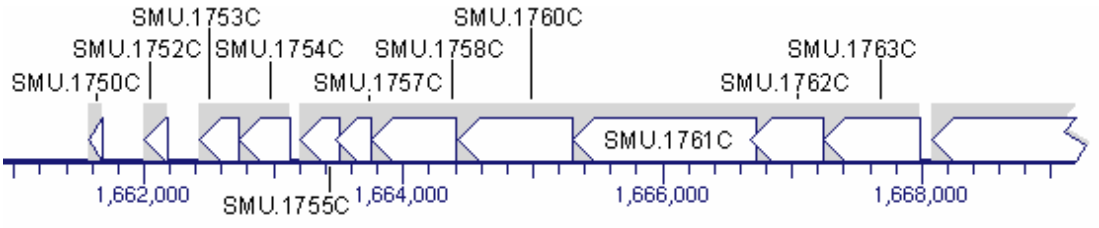


Figure 5.T Section of DNA showing genes SMU.1750C to SMU.1763C This region shows genes up-regulated in dual-species one-day-biofilms that are in close proximity to other up-regulated genes and the surrounding region. Genes SMU.1753c, SMU.1754c and SMU.1757c were up-regulated and they all code for hypothetical proteins linked to replication, recombination and repair.

Three hypothetical proteins were upregulated in dual-species one-day-old biofilms that have a functional classification that predicts they belong in the replication, recombination and repair COG.

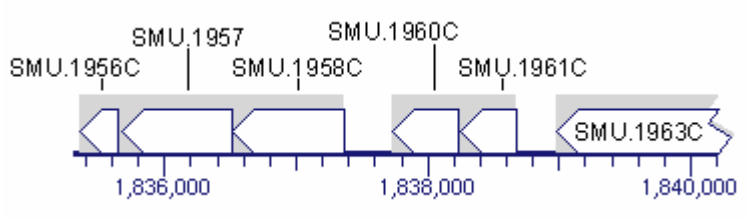


Figure 5.U Section of DNA showing genes SMU.1956 to SMU.1963C This region shows genes down-regulated in dual-species one-day-old biofilms that are in close proximity to other down-regulated genes. Genes SMU.1957, SMU.1958c and SMU.1961c were all down-regulated and these genes all code for putative PTS components. SMU.1957 and SMU. 1958c were also down-regulated in-one-and-three-day-combined dual-species biofilms.

The only cluster of genes that were down-regulated in dual-species one-day-old biofilms is shown in Figure 5.U. These genes are all PTS components, which are very important in regulating carbohydrate metabolism.

5.3.5 Analysis of Gene Expression within the Metabolic Pathways

There are a couple of approaches that can be used to investigate gene expression within metabolic pathways, firstly map expression levels on to the pathway (see Figure 5.V) and secondly to compare a gene list against all pathways to identify

pathways that have significantly more genes that are differentially expressed than would be expected by chance (see Table 5.I). This latter approach is done using hypergeometric probabilities and it takes in to account the total number of genes, the number of genes in the gene list and the number of genes in the pathway. These analyses were conducted using the genes that were identified in the one day, single species versus dual species comparison.

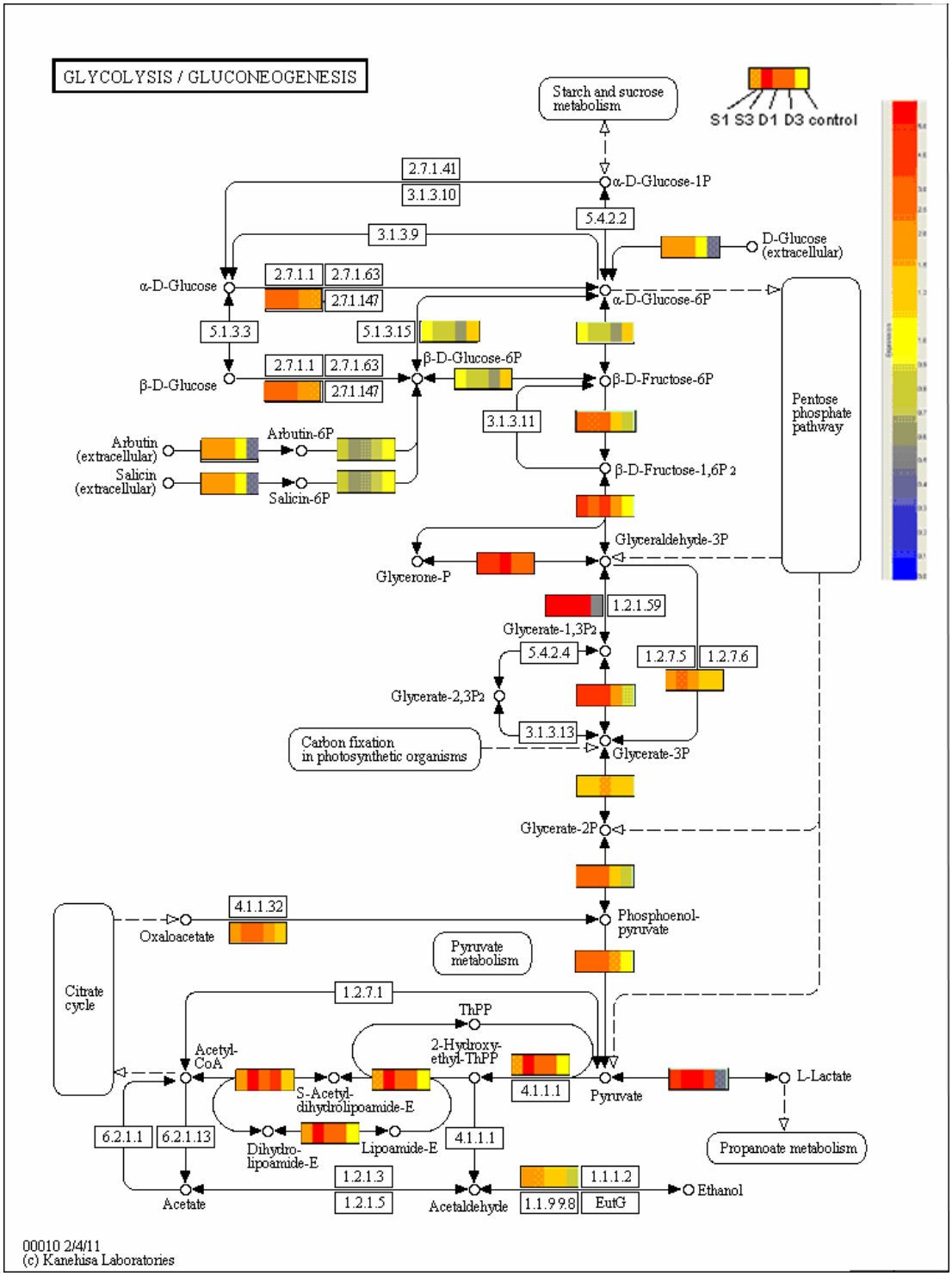


Figure 5.V Glycolysis pathway showing expression of all genes in the different conditions. The key in the top right shows the placement of the different biofilm types in the expression bars; S1:single-species-one-day-old-biofilm, S3:single-species-three-day-old-biofilm, D1:dual-species-one-day-old-biofilm, D3:dual-species-three-day-old-biofilm and control:*S. mutans*-genomic-DNA. (gene list – all genes 1960) Not normalized to any particular channel. Highly expressed genes are shown in red, and genes with little expression are shown in blue.

As Figure 5.V shows un-normalised expression levels, the high levels of expression in the main trunk of the glycolytic pathway are evident.

Table 5.I Identifying Pathways with a significant number of differentially expressed genes.

Sorted ANOVA P<0.05 B+H Single 1d vs. Dual 1d 1.5-fold differences and KEGG PATHWAY – *S. mutans*

Genes overlapping with Pathways	Number of common genes with each pathway	Gene list vs. Pathway random overlap p-value
Glycolysis gluconeogenesis (see Figure 5.W)	6	0.00126
Citrate cycle (TCA cycle)	4	0.00369
Valine, leucine and isoleucine biosynthesis (see Figure 5.X)	4	0.00581
Pyruvate metabolism	4	0.02490
Butanoate metabolism	3	0.02750
Galactose metabolism (see Figure 5.Y)	3	0.03770
Pantothenate and CoA biosynthesis	2	0.08880
Phosphotransferase system (PTS)	3	0.13100
C5-Branched dibasic acid metabolism	1	0.15900
Mismatch repair	2	0.16000
Homologous recombination	2	0.17500
Valine, leucine and isoleucine degradation	1	0.19500
Biosynthesis of steroids	1	0.26200
Carbon fixation in photosynthetic organisms	1	0.32300
Two-component system	2	0.35000
Base excision repair	1	0.35200
DNA replication	1	0.45500
Glycine, serine and threonine metabolism	1	0.52200
Starch and sucrose metabolism	1	0.59900
Phenylalanine, tyrosine and tryptophan biosynthesis	1	0.63200
Fructose and mannose metabolism	1	0.73000
ABC transporters	2	0.82000
Pyrimidine metabolism	1	0.83400
Purine metabolism	1	0.86700

Comparisons were made using the one day single species versus dual species gene dataset (1.5 fold differences) and six pathways were identified as having differentially expressed genes (above the dotted line) but as each of these pathways are part of a large metabolic network, the genes that are differentially expressed can be part of more than one pathway. For example genes SMU.1422 (*pdhB* E1 component β subunit) and SMU.1423 (*pdhA* E1 component α subunit), which code for proteins that break down pyruvate in to S-acetyl-dihydrolipoamide-E (E.C.1.2.4.1) are part of the glycolysis/glucogenesis pathway; the citrate cycle; pyruvate metabolism; valine, leucine and isoleucine biosynthesis and butanoate metabolism. This is a

very interesting reaction as these proteins break down pyruvate into something other than L-lactate which pyruvate can also be broken down into. These genes are up-regulated in dual species biofilms, presumably reducing the amount of L-lactate being produced, and thus reducing the acidification of the environment. To reduce the number of figures required, three pathways can be shown rather than six because the pathways overlap and three can be shown that contain the key enzymatic reactions that are differentially expressed. The glycolysis/glucogenesis; valine, leucine and isoleucine biosynthesis; and galactose metabolism pathways are shown in Figures 5.W to 5.Y.

In Figures 5.W to 5.Y the expression level is normalized to the single species day one channel to better highlight differences in expression (rather than being left un-normalised as in Figure 5.V).

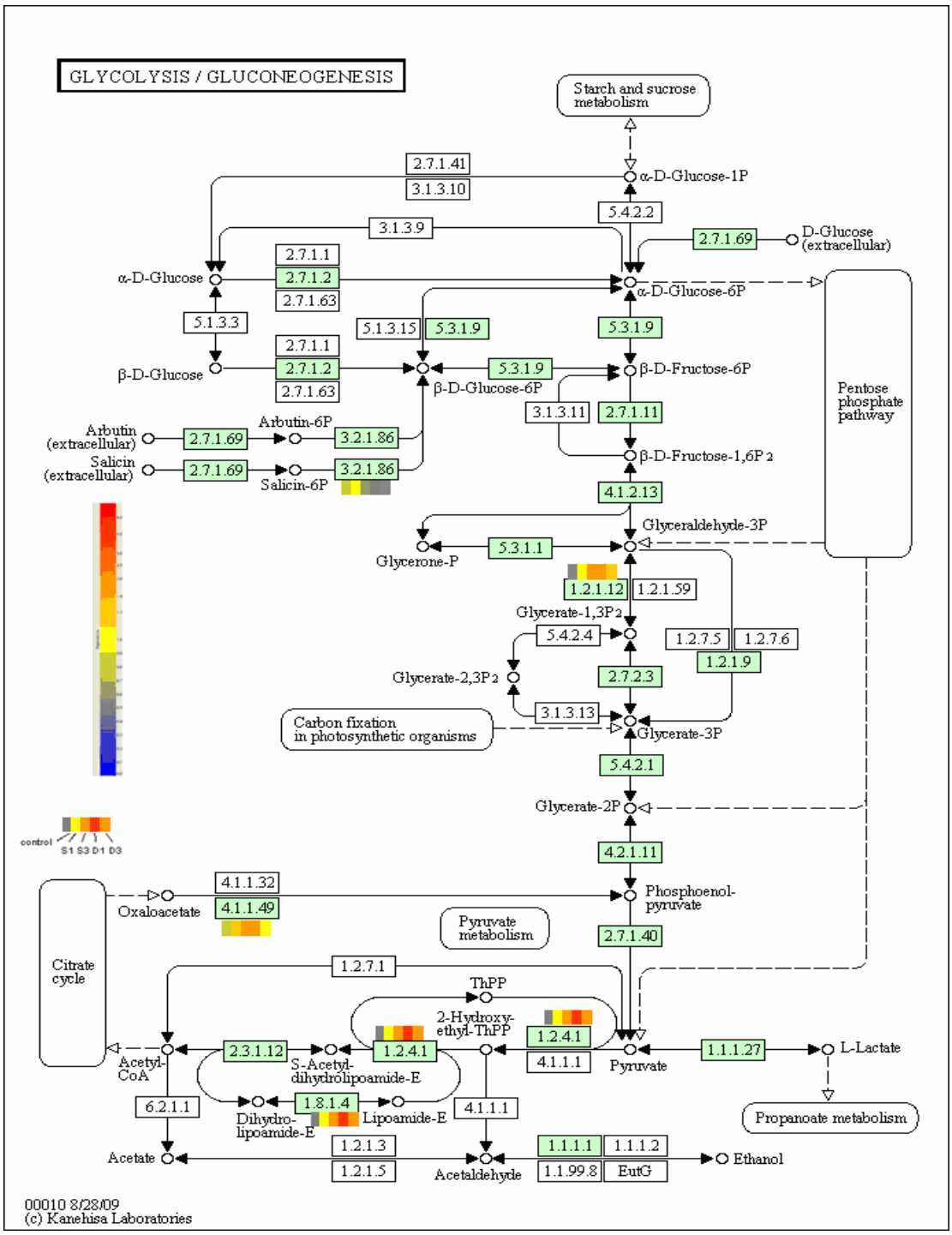


Figure 5.W Differentially expressed genes in the glycolysis pathway.

3.2.1.86 : SMU.1601 celA 6-phospho-glucosidase

1.2.1.12 : SMU.360 GapC glyceraldehyde-3-phosphate dehydrogenase

4.1.1.49 : SMU.1389 pckA hypothetical protein (*also citrate cycle*)

1.2.4.1 : SMU.1422 pdhB E1 component β subunit and SMU.1423 pdhA E1 component α subunit (*also citrate cycle; pyruvate metabolism; valine, leucine and isoleucine biosynthesis; and butanoate metabolism*)

1.8.1.4 : SMU.1424 pdhD putative dihydrolipoamide dehydrogenase (*also citrate cycle and pyruvate metabolism*)

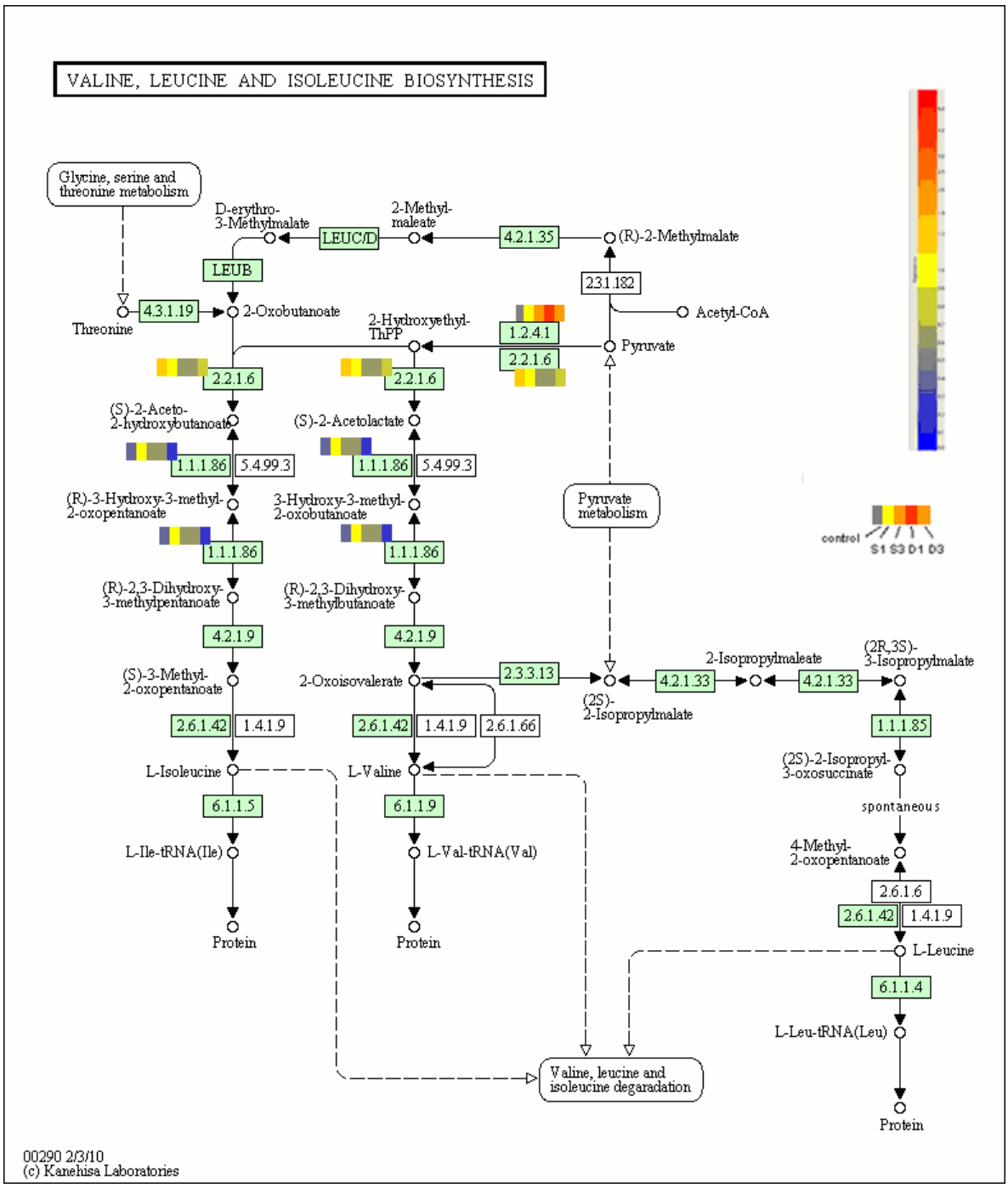


Figure 5.X Differentially expressed genes in the valine, leucine and isoleucine metabolic pathway.

2.2.1.6 : SMU.232 ilvH acetolactate synthase 3 regulatory subunit (*also butanoate metabolism*)

1.1.1.86 : SMU.233 ilvC ketol-acid reductoisomerase

1.2.4.1 : SMU.1422 pdhB E1 component β subunit and SMU.1423 pdhA E1 component α subunit (*also glycolysis/gluconeogenesis, citrate cycle, pyruvate metabolism, and butanoate metabolism*)

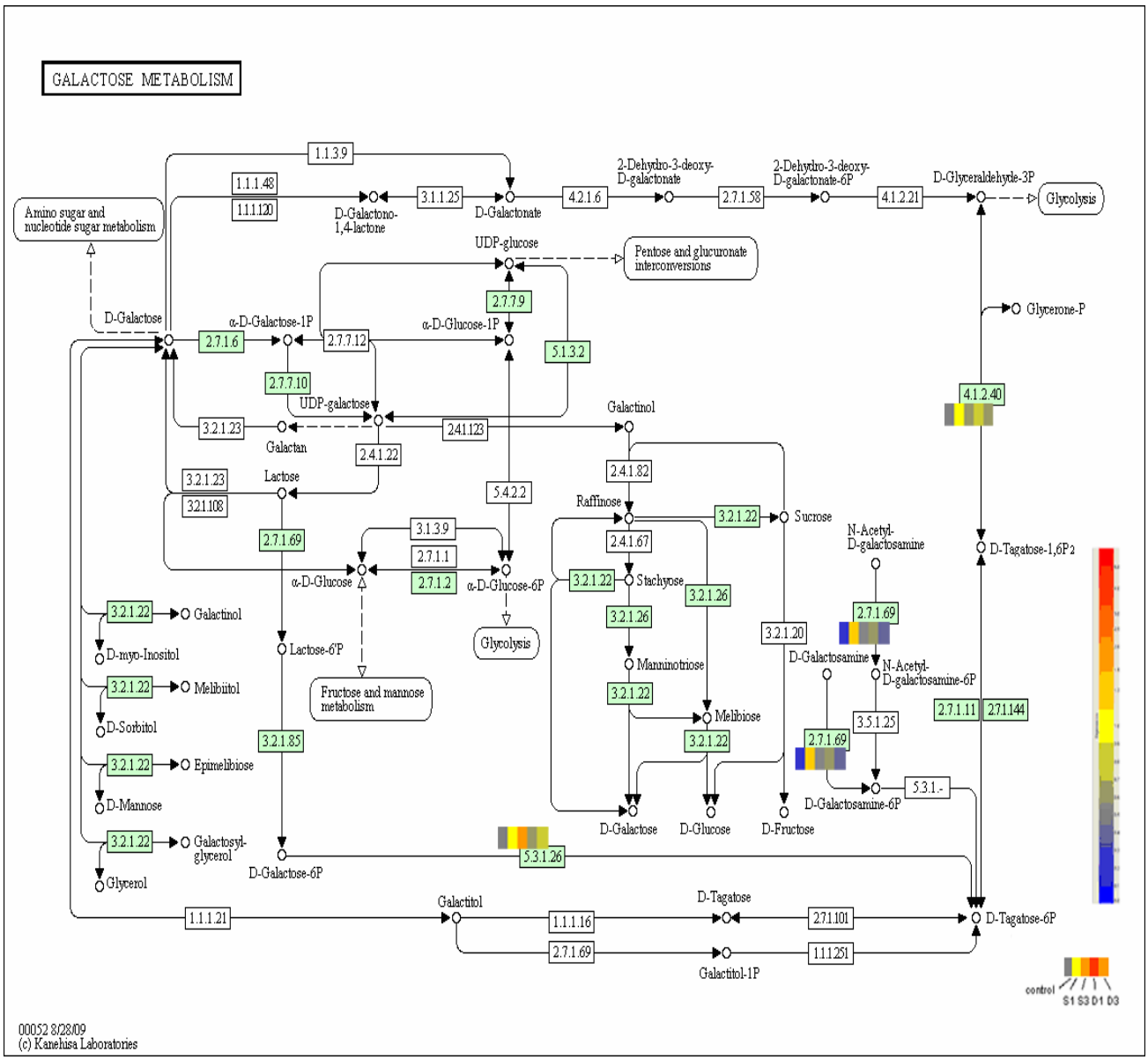


Figure 5.Y Differentially expressed genes in the galactose metabolism pathway.

4.1.2.40 : SMU.116 lacD2 tagalose 1,6-diphosphate aldolase

5.3.1.26 : SMU.1496 lacA galactose-6-phosphate isomerise subunit lacA

2.7.1.69 : SMU.1961c putative PTS system sugar specific EIIa component

5.3.6 Investigation of Genes that are Interesting due to Previous Studies

Of the 14 two-component systems, 3 were found to be differentially expressed: SMU.1128c (up-regulated in dual species 1 day biofilms and up in dual species biofilms), SMU.486 (down-regulated in dual species 1 day biofilms and down in dual species biofilms) and SMU.1916c (up-regulated in dual species 1 day biofilms). SMU.486 and SMU.1128c affect stress tolerance (Biswas et al. 2008) and SMU.1128c mutants are acid sensitive (Ahn et al. 2006, Qi et al. 2004). SMU.1916c

is the putative histidine kinase for the competence regulon. Table 5.J provides a summary of what is occurring in genes that were of a priori interest.

Table 5.J Summary of transcription of key genes.

Lactate dehydrogenase	Highly expressed in all conditions but no differences in expression
Biofilm genes (Table 1 – Shemesh et al. 2007)	Some genes differentially expressed
PTS system (14)	Many genes differentially expressed
Carbon catabolite repression *ptsH* and CcpA transcriptional regulator	*ptsH* and CcpA were not differentially expressed however alternative pyruvate metabolic pathways were up-regulated in dual species biofilms and in the ageing single species biofilm
ABC transporters (4 identified but many putative)	9 putative transporters differentially expressed
Two component systems	3 differentially expressed
Bacteriocins	Mutacins IV and V up-regulated in dual species [one day and combined]
Genetic competence	CSP and ComD up-regulated in dual species biofilms [one day and combined]
Quorum sensing	CSP [one day and combined] and ComD [one day] up-regulated in dual species biofilms
nucleotide transport and metabolism	Guanylate kinase up-regulated in dual species biofilms [combined]
LuxS (autoinducer-2)	No gene expression changes identified
F^0F^1 membrane-bound proton-translocating ATPase	No gene expression changes identified
Nutritional alarmones, RelP and RelRS	No gene expression changes identified
amyB amylase gene promoter	No gene expression changes identified
Stress regulon	ClpP (caseinolytic protease) – two of 5 protein subunits up-regulated in dual species one day old biofilms and two TCS were differentially expressed

5.3.7 Summary of Results

This chapter investigated differences in *S. mutans* gene-expression between single-species *S. mutans* biofilms and dual-species *S. mutans* and *V. dispar* biofilms.

- Novel RNA extraction with enrichment method described in materials and methods produced the best quantity and yield of RNA while it also preferentially enriched for *S. mutans* RNA.
- 83 genes were identified as differentially expressed by *S. mutans* growing in one-day-old single-species biofilms when compared with dual-species (growing with *V. dispar*) biofilms (56 up-regulated and 27 down-regulated in dual species biofilms).
- The age of the biofilm and the resulting environment had more of an effect on *S. mutans* gene expression than co-culture with *V. dispar*.
- Many more genes of unknown function were up-regulated in dual-species one-day-old biofilms than would be expected by chance while there was no significant difference in the number that were down-regulated.
- Very little RNA was recovered in dual-species three-day-old biofilm extractions.
- Alternatives to metabolising pyruvate into lactic acid were up-regulated in aging biofilms.
- SMU.206c, a hypothetical protein, had the highest observed fold-change, an 8.9 fold up-regulation in three-day single-species *S. mutans* biofilms.
- Mutacin V was up-regulated in dual species biofilms.
- There were high levels of expression of genes coding for enzymes in the main trunk of the glycolysis/gluconeogenesis pathway in all biofilms.
- The glycolysis/gluconeogenesis; citrate cycle (TCA cycle); valine, leucine and isoleucine biosynthesis; pyruvate metabolism; butanoate metabolism; and galactose metabolism pathways all had significantly more differential expression within their pathways than would be expected by chance.

5.4 Discussion

5.4.1 Overview

Investigating *S. mutans* at the genetic level allows much deeper insight into the strategies it is employing to grow and survive. This study is similar to one reported in the literature (Luppens et al. 2008) but there was no crossover with the up-regulated genes they identified and only some crossover in the down-regulated genes they identified. This study identified many more differentially expressed genes, most probably from employing an improved method of RNA preservation and extraction (despite having a more stringent biological significance cut-off in some of the comparisons). In their study, to collect the RNA; medium was added, the slurry swabbed, then centrifuged and only then added to RNApro to protect the RNA, with the additional possibility of collecting planktonic as well as biofilm cells. In this study filters with the bacteria growing on them were lifted off the medium and placed immediately in guanidium thiocyanate, which immediately preserved the RNA. RNA degrades rapidly but is also transcribed rapidly, and thus in the Luppens et al. (2008) study some RNA of interest would have been degraded while different artefactual RNA would have been transcribed due to the changing conditions, not least due to the stress caused by exposure to oxygen and the early stages of centrifuging. Furthermore this study enriched for *S. mutans* RNA while in theirs they just doubled the amount of RNA used to account for the extra species in dual species biofilms.

Luppens et al. (2008) used five biological replicated when this study only used four, however this study investigated two time points while they only looked at one. For the common reference, they used a pool of RNA collected from both types of single species biofilms and dual species biofilms while this study used *S. mutans* DNA as the reference. DNA is much more robust and is consistent so while this difference may not have affected the results in the comparison, using DNA allows for the same common reference to be used in different labs and at different times while maintaining an extremely high level of consistency, thus different researchers in a different laboratory could extend this study and directly compare their results. They grew their biofilms at a liquid solid interface while in this study the biofilms were grown at a solid gas interface. Finally they buffered the pH, whereas this study allowed it to change as it would *in situ*. This is the most fundamental difference

between the two studies and would presumably result in many gene expression differences but it also means the two studies complement each other. They also used a different species of veillonellae, *V. parvula*, which is a similar species, that lives in the same habitat, but which may have different interactions with *S. mutans*. These differences in methods employed can easily explain the lack of similarity between the two different studies.

The differences in expression levels (fold change) are small compared to some reported for highly regulated genes in other bacteria (e.g. 100-fold, Wösten et al. 2004) but are similar to expression level differences identified in other studies using this species (e.g. Luppens et al. 2008, Shemesh et al. 2007). Individual genes are not radically altered but there are changes to many genes, and even a two-fold change in such a highly regulated and complex system can have profound effects, as this is the order of change to gene expression levels seen when comparing *S. mutans* growing under biofilm and planktonic conditions (where the 70 most significantly differentially expressed genes had a modal fold change of 2.19 with a maximum fold change of 15.77, Shemesh et al. 2007). Luppens et al. (2008) found similar changes in expression (the 33 genes that had significant changes in expression [only tested those more than 1.3 fold] had a modal fold change of 2.0 and maximum fold change of 7.1). In this study, the maximum fold change observed was an 8.9 fold up-regulation in SMU.206c in the ageing biofilm (SMU.206c is a hypothetical protein that has not been classified that shows some similarity, but with a poor E value [3.8] to 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, a protein involved in terpenoid biosynthesis; terpenoids are sometimes added to proteins so they attach to the cell membrane).

Many hypothetical proteins were identified as being differentially expressed, and notably more were up-regulated than expected by chance in dual species biofilms. As the study of how different bacterial species interact is relatively new, it is possible that the disproportionately high number of hypothetical proteins of unknown function that are up-regulated in dual-species biofilms serve some function specifically to do with interacting with other species rather than as a consequence of differences in the environment another species creates. That there is no significant difference from chance in the number of hypothetical genes down-regulated further supports that some of the up-regulated hypothetical genes may be ‘interaction genes’. These

possible interaction genes would make interesting candidates for further study using bioinformatic analyses and expression and treatment studies having first validated the expression differences using qRT-PCR. Very few genes involved in interactions have been identified with some notable exceptions such as bacteriocins. However, some of these genes were hypothetical at the time of original annotation and have not been updated since, despite having their function determined (e.g. SMU.150 and SMU.1914c are both still classified as hypothetical proteins despite being identified in the literature as Mutacin IV and Mutacin V [Qi et al. 2001, Hale et al. 2005]).

Some of the more interesting genes to differ in expression included PTS genes, endonucleases, the competence stimulating peptide, bacteriocins, pyruvate dehydrogenase genes, the chromosomal replication protein, amino acid synthases, and glycolysis genes and these are discussed in context in the following subsections which focus on acidogenicity and aciduricity, energy and nutrient acquisition, bacteriocins, competence, the ageing biofilm, conflict and cooperation, strategies for control, and avenues for possible future work.

5.4.2 Acidogenicity and Aciduricity: Hegemony and Survival

S. mutans is primarily of interest as an oral pathogen because it is acidogenic and the acid it produces causes caries. As *S. mutans* lives in an environment where the acid can accumulate despite being periodically washed with saliva, it needs to be able to survive at low pH and it employs multiple strategies to be aciduric. *S. mutans* extrudes protons using the F^0F^1 membrane-bound proton-translocating ATPase, modulates the production of acidic end-products of metabolism, and employs branched chain amino acid biosynthesis (Len et al. 2004, Lemos and Burne 2008). The majority of species in the oral cavity are not aciduric so *S. mutans'* combination of acidogenicity and aciduricity not only allows it to rapidly metabolise a periodically abundant nutritional source and survive in the resulting toxic waste products, but it also allows *S. mutans* to kill and competitively exclude most other species with the ensuing low pH.

Other acidogenic and aciduric species exist within the oral cavity but even the other streptococcal species employ different aciduric mechanisms. Some of these other acidogenic and aciduric species have been linked to caries (Russell 2008) but *S.*

mutans is the primary causative agent. Bradshaw et al. (1989) found *V. dispar* and *S. mutans* increased in relative and total abundance within the biofilm after pulses of sugar. As *S. mutans* can not exclude other acidogenic and aciduric by producing acidic waste products (unless they are less aciduric), it can target them with bacteriocins and this is discussed in Section 5.4.4.

Lactate dehydrogenase which converts pyruvate into lactic acid was highly expressed in all conditions and did not differ significantly in expression. However, dual species biofilms did differ in their expression of pathways that process pyruvate into products other than lactic acid. Parts of the pathways that convert pyruvate into acetyl-CoA, acetate, acetaldehyde and branched chain amino acids were upregulated in dual species one day old biofilms compared with single species one day old biofilms. This same pattern was observed in three day single species biofilms when compared with one day single species biofilms, suggesting that *S. mutans* in dual species biofilms starts to process pyruvate to less acidic, or non acidic products earlier than when it is growing as a single species. This could be as a result of a quorum sensing signal that is shared between *S. mutans* and *V. dispar* or because of a signal generated by *V. dispar*. In addition it shows how metabolic pathways are fine tuned by the control of the expression of RNA to respond to changing environmental conditions and the presence of signals.

S. mutans maintains its internal pH slightly above 7 in optimal conditions, but can tolerate it decreasing, however in the earlier buffering studies, *S. mutans* still needed to extrude the protons from its cytoplasm to maintain intracellular pH. Thus it was still limited by the production of acidity, in that it needed to be producing enough ATP to power the F^0F^1 ATPase pump to extrude the protons.

The strategy of being acidogenic and aciduric is what makes *S. mutans* pathogenic as the acid causes caries, in addition to excluding other species. Its ability to survive and effect the rapid acidification of the environment when carbohydrates are abundant provides a strong selective advantage over less-aciduric species. This powerful selective force overwhelms any selective advantage of cooperating with veillonellae that metabolise lactic acid making the environment less neutral and allowing the production of more lactic acid.

5.4.3 Energy and Nutrient Acquisition

S. mutans has evolved to compete for and effectively utilise the resources it has available. The feast or famine existence in the oral cavity has led to the maximisation of the use of available substrates. Sugars are the primary energy source for *S. mutans*, and these are broken down by glycolysis.

$$\text{glucose} + 2\ NAD^+ + 2\ ADP + 2\ P_i \rightarrow 2\ \text{pyruvate} + 2\ NADH + 2\ H^+ + 2\ ATP + 2\ H_2O$$

However, for glycolysis to function properly, *S. mutans* needs a method to convert NADH back to NAD^+. This is normally done by oxidative phosphorylation but *S. mutans* lacks a functional electron transport chain and cannot do this. It converts the NADH back to NAD^+ using a process called lactic acid fermentation which does not require oxygen or an electron transport chain but rather involves substrate level phosphorylation. This reaction is catalysed by the enzyme lactate dehydrogenase (SMU.1115) which did not show any differences in expression in any of the conditions but it was expressed highly throughout.

$$\text{pyruvate} + NADH + H^+ \rightarrow \text{lactate} + NAD^+$$

This conversion of NADH back to NAD^+ ties the production of lactic acid to energy consumption. While the production of lactic acid is environmentally beneficial to *S. mutans* as it competitively excludes other species, *S. mutans* is also limited by pH. However, alternative pathways exist where pyruvate is broken down using an incomplete TCA cycle, converted to diacetyl, converted to formate (generating an ATP) or ethanol (converting 2 NADH to 2 NAD^+) using the pyruvate formate-lyase pathway or converted using branched chain amino acid biosynthesis (Len et al. 2004).

The genes for the incomplete TCA cycle are up-regulated in both dual species biofilms and ageing biofilms while the genes for branched chain amino acid biosynthesis are down-regulated in dual species one day biofilms. The genes for the production of acetate and ethanol are not differentially expressed between the different conditions. *S. mutans* continues to produce lactate in all the conditions but appears to employ alternative pathways to breakdown pyruvate as the biofilm ages

and once the environment is already very acidic. It also increases the expression of genes for the TCA cycle in the one day dual species biofilms, which presumably should reduce the rate of production of acid in dual species biofilms. The earlier investigation reported in Section 3 showed that in dual species biofilms after one day the environment was already more acidic than single species biofilms but not significantly so, therefore it is unlikely that the increased use of this less acidogenic pathway is due to a cooperative behaviour to benefit *V. dispar* but is possibly as a result of a slowdown of the production of acid induced by *S. mutans* own tolerance of acidity. Genes involved in branched chain amino acid biosynthesis, *ilvC* and *ilvH*, are downregulated in dual species one day old biofilms indicating that it is likely pyruvate is being converted to ethanol and acetate despite there being no observed change in expression of alcohol-acetaldehyde dehydrogenase (*adhE*), the bifunctional enzyme that catalyses both reactions. Len et al. (2004) found levels of formate, acetate and particularly ethanol were markedly increased when comparing *S. mutans* growing in continuous culture at pH 5 compared with when it was grown at pH 7. Using *adhE S. mutans* can adjust NADH oxidation as required (as breaking pyruvate down to ethanol converts two NADH to two NAD^+ and producing acetate produces an ATP). One of the three genes (*aldB*, putative alpha-acetolactate decarboxylase) involved in the conversion of pyruvate to diacetyl is up-regulated in three day single species *S. mutans* biofilms indicating *S. mutans* is employing multiple non lactate dehydrogenase pathways to process pyruvate once the environment is already acidic.

Similar results were found in a study investigating a CcpA mutant (Abranches et al. 2008). CcpA is a transcriptional regulator involved in carbon catabolite repression and virulence, and one of its functions is to regulate metabolism so that carbohydrates are primarily moved through lactate dehydrogenase when they are abundant but when they are limiting similar gene expression changes are observed to those described above. Section 3.3.3.3.2 indicates that there would have been a surplus of carbohydrate throughout this experiment, thus *S. mutans* will preferentially process carbohydrate to lactic acid when carbohydrates are abundant and the environment is not too acidic, but if carbohydrates are limiting or the acidity of the environment is nearing critical levels for *S. mutans* tolerance, then the other metabolic pathways are employed.

The annotation of the genome can cause complications in deciphering what expression differences actually mean, as demonstrated by this example. Dual species biofilms had putative mannose PTS genes downregulated, specifically the putative $EIIC^{Man}$ and $EIID^{Man}$ domains. The mannose PTS is involved in the uptake of glucose but also has many profound effects on CCR, PTS activity, biofilm formation and genetic competence, although this has been linked to the $EIIAB^{Man}$ domains (Abranches et al. 2006). The putative $EIIC^{Man}$ and $EIID^{Man}$ domains were downregulated in dual species biofilms. These domains have been linked with mutacin I expression when they are downregulated (Nguyen et al. 2009). There are two different regions with homologous genes within the genome that are classified as putative mannose PTS genes and only one region differed in this study, and importantly it is not the one mentioned in either the Abranches et al. 2006 or the Ngyuyen at al. 2009 study. Ajdić and Pham (2007) found that this operon was upregulated in the presence of fructose and have proposed it is an $EII^{Fru/Man}$ locus inducible fructose EII transporter so it is unlikely the homology with the other mannose PTS genes would cause there to be any of the differences identified by Abranches et al. (2006) or Ngyuyen at al. (2009). There was no fructose in the medium in any of the studies so the difference in expression must be tied to a signalling pathway initiated by the presence of *V. dispar* or, more probably, its metabolites.

Many amino acid synthases were down regulated in the older biofilms, indicating that there was less growth and consequently less need for amino acids, or that there were more available from the environment (released in the breakdown of the non-viable bacteria detected in Chapters 3 and 4), or more probably a combination of both of these reasons.

5.4.4 DNA attack and Bacteriocins: Clonal Sacrifice, Calibration Error or Genocide

Dual species biofilms are up-regulating proteins associated with defence including endonucleases, a DNA methylase and two bacteriocins (mutacin IV and mutacin V as well as its probable associated proteins). Focusing on the bacteriocins, there are three possible reasons for this; clonal sacrifice to increase the chances of survival for those remaining, calibration error where resources are being wasted in the production of products that in the circumstances are of no use, or genocide of *V. dispar* to free up space or resources. These will now be explained in more detail. The population of

S. mutans in each biofilm is clonal with any genetic differences due to mutations that have occurred during the experiment. However individual bacteria could be expressing different phenotypes, with some bacteria expressing immunity genes while others are not, so a proportion of the population could be susceptible at any given time. As nutrients and space become limiting, this subset of the population could be sacrificed for the benefit of those remaining. Alternatively, the production of bacteriocins could be a costly and useless blunder initiated by the triggering of signalling mechanisms that have never needed to be calibrated to such a precise level, as in *S. mutans'* natural environment such triggering would be beneficial. The final possibility is that the bacteriocins are being produced to kill *V. dispar*. The literature states bacteriocins typically target closely related species (Eijsink et al. 2002, Dawid et al. 2007), and consequently bacteriocins are normally only tested against closely related species (eg. Hale et al. 2005). Hale et al. (2005) identified and named mutacin V, (SMU.1914c which is upregulated in dual-species biofilms in this study) after testing it on *Streptococcus* species, *L. lactis* and *Micrococcus luteus* and found it was mainly active against nonstreptococcal targets. They only tested two non-streptococcal species and did not test any Gram-negative species. Hossain and Biswas (2011) followed up this research and identified mutacin IV and V are active against multiple *Streptococcus* species in contrast to the results of Hale et al. (2005).

There are a number of genes in the same region of the genome as mutacin V that are also upregulated in dual species biofilms that have not had their function defined. These, when investigated for sequence similarity to other proteins using protein BLAST searches, show similarity to bacteriocins and bacteriocin associated proteins (control, production, immunity and export) and all of these are immediately adjacent to genes involved in genetic competence and this is elaborated on in the next section (5.4.5). Some bacteriocins have multiple protein subunits (display quaternary structure) and some of the other expressed proteins may be other bacteriocin subunits. Hale et al. (2005) tested these and found no effect but they only tested them against closely-related species. Of the three possible explanations for the up-regulation of bacteriocin production in dual species biofilms, the first clonal sacrifice is possible if the signal is related to total cell density in the biofilm as the dual species biofilms comprise many more bacteria. The dual species biofilms would have more signalling molecules (often metabolites) and these presumably are triggering the production of mutacin V. If mutacin V has no effect on *V. dispar* then it is calibration error and if it

does have an effect then it is genocide that is causing the up-regulation of mutacin V in dual species biofilms. Despite bacteriocins only being tested on closely related species it is unlikely that mutacin V is active against *V. dispar* as in the buffered planktonic study (Section 3.3.2.2.1) there was no indication of *V. dispar* having lower total viable counts when growing as a dual species rather than as a single species indicating that it is not killed by mutacin V (although this assumes that mutacin V is being upregulated in dual-species planktonic cultures). Hence it appears that the upregulation of mutacin V by *S. mutans* in dual species cultures is probably a calibration error.

Investigating signalling mechanisms and bacteriocins in the literature identified that the production of many bacteriocin products is downregulated in the absence of ClpP (caseinolytic protease) a heat shock protein (Chattoraj et al. 2010). ClpP comprises five protein subunits, two of which are upregulated in dual species one day old biofilms relative to single species one day old biofilms. Thus the presence of ClpP would lead to the relative downregulation of bacteriocins in single species one day old biofilms relative to the dual species one day old biofilms. The upregulation of ClpP in the dual species one day old biofilms could result from any of a number of stresses caused from the increased complexity of the environment due to the presence of another species. Also, Kreth et al. (2007) identified potential ComE binding sites in the promoter regions of both mutacin IV and mutacin V. ComE is part of the signalling system responsible for genetic competence. The competence stimulating peptide CSP is produced by the gene SMU.1915 which was upregulated in dual species biofilms. CSP is recognised by the two component signalling system comprising ComD and ComE. ComD, but not ComE was observed to be upregulated in dual species biofilms. While ComE is not upregulated, the putative binding sites in the promoters of the bacteriocins, and the upregulation of SMU.1915 and ComD indicate that the bacteriocins are likely upregulated as a result of quorum sensing and competence.

5.4.5 Competence: Acquiring New Genes, Food or Both?

Competence, the uptake of DNA into the cell, is well described as a mechanism to acquire DNA for new genes and increase diversity with the gene pool. Many studies have shown the horizontal transfer of antibiotic resistance and metabolic

enzymes (reviewed in Syvanen and Kado 1998). However, it has recently been postulated that bacteria (Finkel and Kolter 2001) may be acquiring DNA as a nutritional source. Guiral et al. (2005) found that the expression of cell lysis genes, bacteriocins and competence genes is coupled in *S. pneumoniae*. In *S. pneumoniae* competence genes are controlled by the two component system ComED and bacteriocin genes are controlled by the two component system BlpRH while in *S. mutans* both competence and bacteriocin genes may be controlled by ComED (Kreth et al. 2007).

The coupling of cell lysis genes, bacteriocins and competence genes in *S. pneumoniae* and the possible coupling of the signalling mechanism in *S. mutans* does not mean that the DNA is going to be used as food, as the bacteria could be aggressively acquiring new genetic material for diversity or repair. Mutacin V and other putative bacteriocin related proteins were upregulated in dual species biofilms, and these genes are adjacent to the competence genes *comD* and *comE*. The autolysin AltA did not differ in expression in the different conditions and no genes with sequence homology to *lytA* (*S. pneumoniae* gene associated with competence) differed in gene expression. The gene for the enzyme guanylate kinase (*gmk*) is the only gene involved in nucleotide metabolism (to metabolise the DNA) that was upregulated in dual species biofilms.

It would be interesting to investigate the use of DNA as a food source in energy limited experiments, and to identify if the putative bacteriocin genes around mutacin V are upregulated (using qRT-PCR) when the bacteria are stimulated with CSP (competence stimulating peptide). Diversity allows bacteria to persist in ever-changing environments, and as *S. mutans* is naturally competent it can gain or re-acquire DNA as and when required. It is probable that DNA is taken up to increase diversity or to repair damaged DNA (Claverys et al. 2006) but given the feast or famine existence of *S. mutans*, it is also possible that DNA would be useful nutritionally in times of famine. Perry (2009) argues that competence does not exist solely to provide food as bacteria can become competent in nutrient rich conditions, take up DNA that has been degraded to be single stranded (thus losing out on half the food), and could potentially excrete a non-specific nuclease and uptake the nucleolytic products, a simpler nutritional strategy (as *Bacillus subtilis* does, Dubnau 1999). However, DNA is clearly useful to increase diversity and to repair DNA and if it is to

be used nutritionally, the mechanisms, even if not optimal, already exist to uptake single stranded DNA and this can be seconded in to act as a nutritional mechanism. Finally, *S. mutans* growing as a biofilm could not break DNA down to nucleotides and then import them without interfering with the structural integrity of the EPS as the DNA polymers are an important part of the biofilm scaffold (Steinberger and Holden 2005, Moscoso et al. 2006, Flemming et al. 2007).

Producing bacteriocins when they are not needed can be considered as signalling error, however *S. mutans* has a small genome and streamlined signalling mechanisms. It may have evolved a general stress response, that involves the heat shock protein ClpP (mentioned above), that causes it to produce bacteriocins and to become competent to increase genetic diversity and to repair damaged DNA.

5.4.6 The Ageing Biofilm

The ageing biofilms needed to respond to diffusion limitations, increasing acidity, and the increasing spectrum of substrates. Many gene expression changes occurred indicating the changing nutritional strategies of the ageing biofilm, including *adh* and *pdh* genes (which are involved in the metabolism of pyruvate), which were strongly up-regulated thus providing further ways to get additional energy from pyruvate, without converting it to lactic acid. Once acidity started limiting *S. mutans* it switched to employ alternative metabolic pathways. This supports the early work of Mikx and van der Hoeven (1975) who identified that when there was little glucose and few *S. mutans* present, lactic acid was the predominant waste product but when there was abundant glucose and many *S. mutans* the predominant waste product would switch to acetate.

NADH oxidase was up-regulated in three day biofilms which is a peroxide forming enzyme that deals with oxidative stress. It is a good way to convert NADH back to NAD+ but it should not have been able to function without oxygen in a supposedly anaerobic chamber. It may, however, have been up-regulated in response to some other signal as part of a stress response. Amino acid synthases were down-regulated indicating the amino acids were available in the environment, or that the bacteria no longer needed them as much, or most likely a combination of these two reasons. A wall integrity membrane protein was up-regulated in the older biofilm giving an

indication of the increasing stresses on the bacteria. Glycogen phosphorylase (*glgP*) was up-regulated, which indicated *S. mutans* was accessing its energy reserves, as in times of abundance it converts glucose to glycogen as an energy store. Taken together these changes indicate *S. mutans* in the three day biofilm had switched from a rapid growth to a survival strategy.

5.4.7 Do they Actually Cooperate?

S. mutans and *V. dispar* were investigated because they are predicted to cooperate because of their association with lactic acid (van der Hoeven et al. 1978, McBride and van der Hoeven 1981, Mikx et al. 1972, Mikx and van der Hoeven 1975, Kara et al. 2006, Palmer et al. 2006, Chalmers et al. 2008) but do they actually cooperate? The original hypothesis was that *S. mutans* and *V. dispar* would both take action to benefit each other. Certainly, as the biofilm gets older and as the environment is acidified, they do not appear to. However, initially both species seemed to have higher viable counts than when growing alone as demonstrated by the higher total viable counts shown in Section 3. The plentiful, but not too plentiful, lactic acid at these early stages can explain the benefit to *V. dispar*. *S. mutans* gains some benefit from the removal of lactic acid, as it was observed to grow best at a neutral pH in the buffered experiments, so this can explain the sometimes observed benefit of co-culture with *V. dispar*. However the acidification of the environment is actually useful to *S. mutans* as this gives it a competitive advantage.

Many hypothetical proteins were up-regulated in the dual species biofilms, and these could be specifically for interacting with the other species, either in a competitive or cooperative way. In a complete frame shift of the original hypothesis, actually removing the lactic acid could be a competitive act against *S. mutans* rather than a cooperative act because the acid competitively excludes so many species. With *S. mutans* more interested in creating an environment favourable to itself and little else, the onus is on *V. dispar* to drive the interaction. However, in a similar study using *S. gordonii* and *V. atypica* (Egland et al. 2004) there was signalling between the two species resulting in *S. gordonii* upregulating *amyB* which actually leads to an increase in the production of acid. *S. mutans* and *V. dispar* benefit each other but only in a narrow range of conditions indicating how interactions can change over time.

5.4.8 Strategies for control

Lactic acid is the problem, and any method that tricks *S. mutans* in to employing alternative methods to source energy from pyruvate without breaking it down to lactic acid (excluding the production of formic acid which is actually a stronger acid) would be beneficial in the prevention of caries. This could be done by subverting the carbon catabolite repression signalling pathway. Alternatively, short circuiting sensing mechanisms that allow acidogenic organisms to survive at low pH may be more useful in combating caries than targeting specific enzymes as multiple species are involved with different enzymes but similar sensing methods (Burne et al. 2009).

5.4.9 Future work

The differential lysis did not lyse all the *V. dispar* cells and an experiment could be carried out in which the concentration of guanidine cyanate is increased to determine if there is a concentration that lyses all of the *V. dispar* but none or very few of the *S. mutans* and this could be checked microscopically using Gram staining. The three day dual species data was poor and could be improved given time and funding by repeating this part of the experiment and increasing the size of the pool of filters to increase the amount of mRNA. The expression level differences of key genes could be investigated and validated using qRT-PCR. Mutacin IV and mutacin V could be tested on *V. dispar* to determine if they have any effect (although it is unlikely they do). The use of competence as a nutritional strategy could be investigated using nutrient limited studies. Also, when the facilities allow, studies like this can include an analysis of the remaining products which may help elucidate differences in nutritional strategies. Other *S. mutans* strains are being sequenced (e.g. Maruyama et al. 2009) and comparative bioinformatic studies can be conducted. Multispecies bacterial biofilms will be increasingly studied, and these studies will include increasing numbers of species.

The number of bacterial species that have had their genome sequenced is growing so soon studies will be done where multispecies communities are studied using whole genome transcriptomic analyses where all the species in the community have genome markers for their entire genome on a single microarray. Finally, the excellent work done by Ajdić et al. 2001 in sequencing the genome has not been followed up by maintaining an accurate annotation of the function of the genes as often the

function is known but not updated in the online databases. All scientists using the genome data, and increasing the understanding of the function of individual genes need to update the genome data rather than simply identifying function in scientific journals.

6 Modelling Biological Complexity and the Modelling of Bacterial Biofilms

6.1 Introduction

Models are a simplified representation of a system that can identify the fundamental mechanisms that are operating and the way they are linked. Quantifying a system in mathematical terms and relationships is a way of testing if the understanding of the system is valid, as it should reproduce the relevant phenomena. The most important aspect of biological mathematical modelling is that it can be used to develop testable hypotheses and trial experimental design. A mathematical model was developed to investigate the key components of the *S. mutans*, *V. dispar* and lactic acid system and the way these components interact within this system. This model was then used to investigate a number of scenarios.

The newly developed non-monotonic growth model, described in the methods section of this chapter (Sections 6.2.1.2 and 6.2.1.3) combines the form of the predator prey equation (see Section 1.12.3), by adding an additional equation for the concentration of lactic acid (rather than one species predating on the other), with the structure of the exponential growth and carrying capacity of the logistic equation to model the two (and as shall be seen a third) species.

6.1.1 Aims

The aim of this chapter was to model the growth, interaction and decline of two bacterial species that are growing together as a biofilm and are coupled together by a metabolic network. The intent was to develop a model that captures not just the growth and decline of each of the individual species but also sensibly couples the dynamics of the two species together, reflecting and highlighting the nature of their interaction. A key intention was to get data about how the nature of the relationship changes emerging from the model, rather than entering parameters identifying these changes into the model. In other words points of inflexion in the growth curves are emergent properties of the model rather than parameters entered into it. This model was developed to improve the understanding of the factors within the system and how they relate to each other. Thus the aims were:

(i) to integrate the information collected into a systems biology model of the interactions of *S. mutans* and *V. dispar*; two key species of the dental plaque biofilm.

(ii) to use this model to test the validity of our understanding of the system.

(iii) to apply this model to identify ways to beneficially modulate this biofilm.

6.2 Materials and Methods

The model was designed and executed using the software Wolfram Mathematica 6 (version 6.0.1.0). The non-monotonic growth model was developed by combining and extending the logistic growth model and the Lotka-Volterra predator prey model.

6.3 Results

This section shows the output from running the non-monotonic growth model using a range of parameters, and the implementation of a different model form to inform how a modified non-monotonic growth model could be constructed to better fit the data.

6.3.1 Non-Monotonic Growth Model

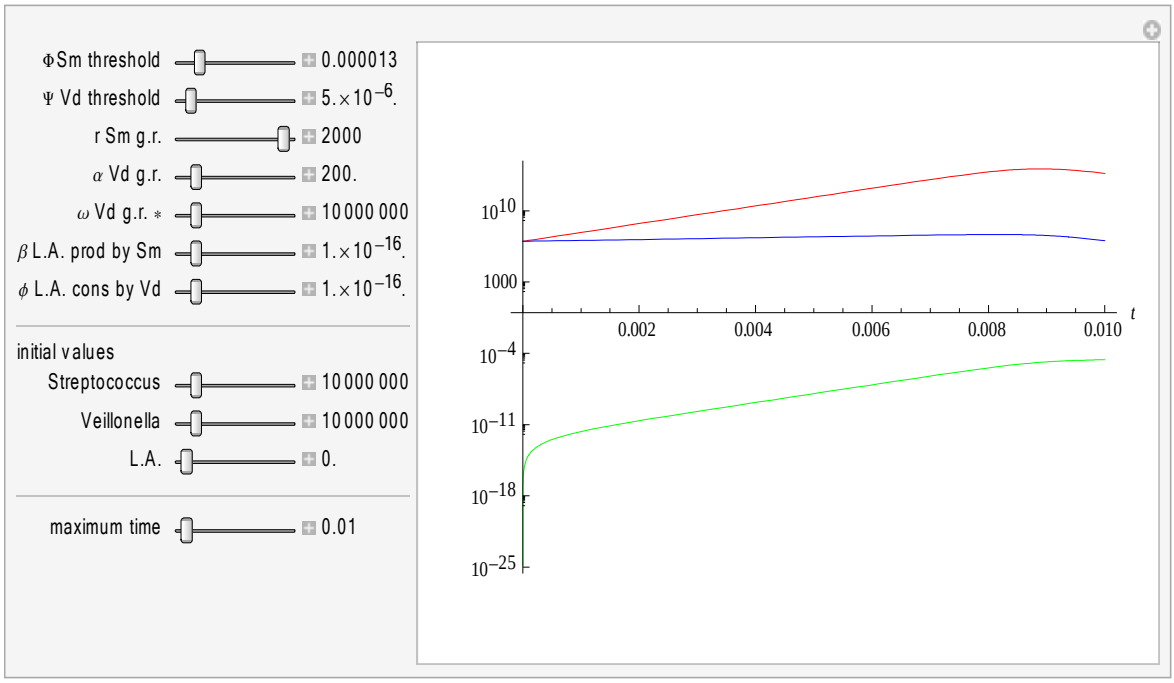


Figure 6.A Output of non-monotonic growth model using experimentally calculated parameters. The box on the right shows the output of the model. The population of *S. mutans* is shown in red and the population of *V. dispar* is shown in blue. The concentration of lactic acid is shown in green. The left third of the figure shows the parameters and slider bars that can be used to adjust the value of the parameter, and to the right of the slider bar, the value used for that parameter at the start in the model is shown.

Figure 6.A shows the first run of the model which used the parameters that were set or measured in the experiments (Chapter 3). In this run the maximum peak for both *S. mutans* and *V. dispar* is reached after 0.009 days; much earlier than the experimental results. This implies that the parameters used in the model, and/or the model itself are wrong, or at least not sufficient to recreate the experimental results

accurately. In the experiments (Chapter 3), after one day of growth both species have grown massively in numbers and have stopped growing exponentially, and thus it is very difficult to measure their growth rate as a biofilm. The values of 2000 and 200 are much lower than they would be for uninhibited exponential growth over a day but they are still too large for this model. The model itself does not accurately recreate the experimental results but this does not rule out its usefulness as a tool to investigate the system and conduct experiments *in silico*. Thus the parameters of the model were changed in an attempt to use it as a totally dimensionless model to investigate the dynamics of this system.

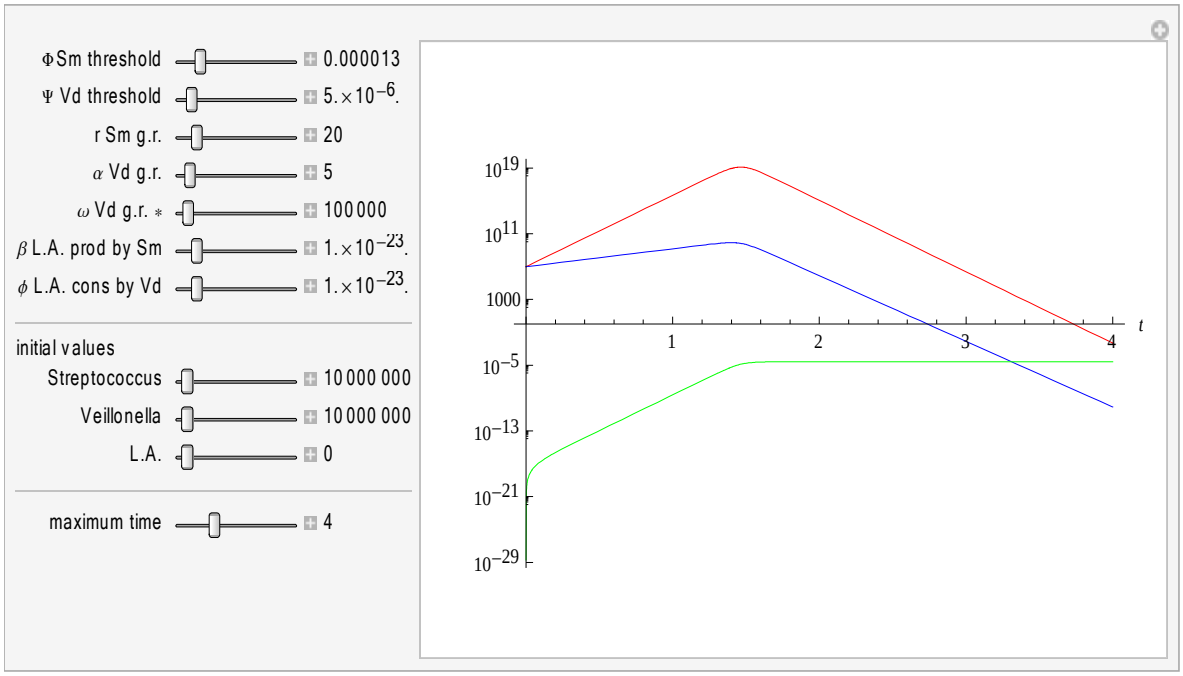


Figure 6.B Output of non-monotonic growth model with parameters changed to produce non-dimensional model. The values of the new parameters are shown on the left of the figure. The population of *S. mutans* is shown in red and *V. dispar* is shown in blue. The concentration of lactic acid is shown in green.

Figure 6.B shows the logarithmic increase of *S. mutans*, *V. dispar* and lactic acid until t=1.5, when lactic acid reaches the critical threshold for *S. mutans*, at which point the lactic acid concentration levels off, and *S. mutans* and *V. dispar* die off logarithmically. This pattern of growth is similar to that observed experimentally. An interesting point to note from this is how shortly after lactic acid reaches the critical threshold for *V. dispar*, it reaches the critical threshold for *S. mutans*.

This model implies that these two species are coupled around lactic acid, and this relationship can describe much of the pattern of growth that is observed experimentally. The model is far from a perfect fit, and the parameters needed to be changed; so it could be improved by including other processes in the model, and tweaking the form of the model, but it is suitable to use to explore the dynamics of the system.

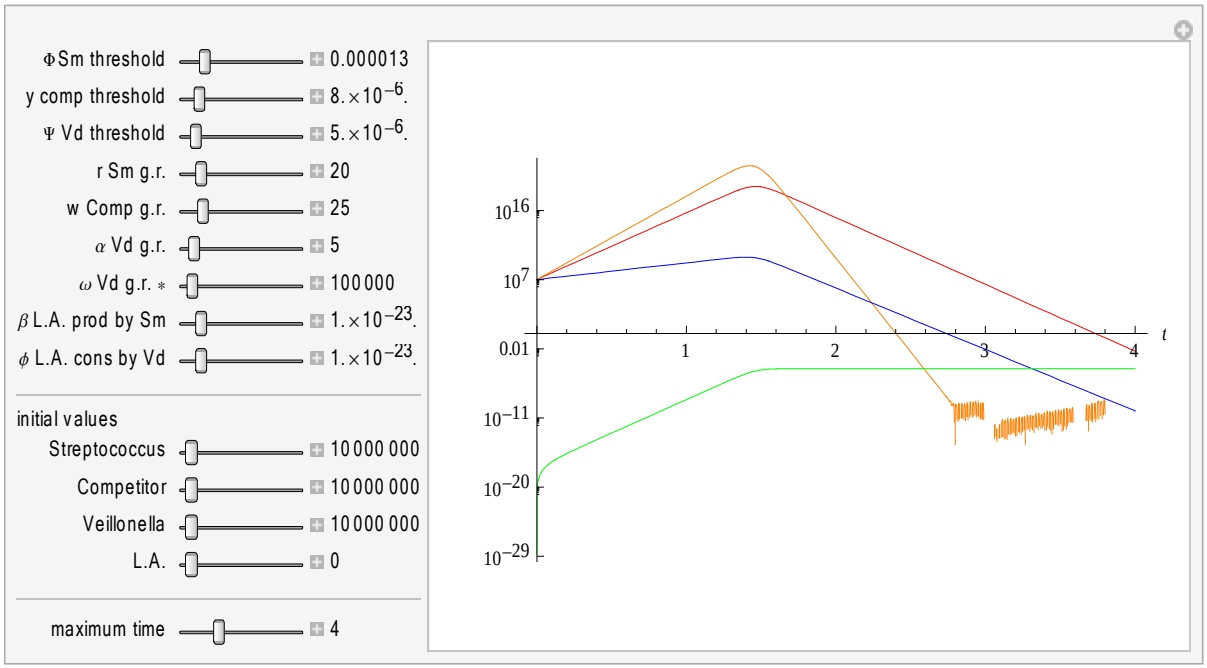


Figure 6.C Introduction of a competitor to the system. This figure shows the introduction of a competitor in yellow, with the new parameters for this competitor included on the left. The growth rate of the competitor is faster but it is set to be less tolerant of acidity than *S. mutans*. The populations of *S. mutans* and *V. dispar* are shown in red and blue. The concentration of lactic acid is shown in green.

Figure 6.C shows a run of the model where a competitor of *S. mutans* has been introduced to the biofilm. This competitor was chosen to have the same growth equation as *S. mutans* but it does not produce lactic acid, although it is susceptible to it. In this run, the competitor was chosen to have a faster growth rate than *S. mutans* but a critical threshold between that of *S. mutans* and *V. dispar*. The competitor grows exponentially before reaching its threshold and dying rapidly. The lactic acid concentration and rate of change show the pH can potentially change very rapidly, but also that the critical threshold for *S. mutans* is reached very shortly after it is reached for *V. dispar* and for the competitor. However, it shows that *S. mutans* can produce acid to outcompete (despite dying itself) competitors. In situ, other factors

(e.g. washing and buffering effects of saliva) would likely raise the pH before all the *S. mutans* died. It demonstrates that *S. mutans* could be using its acidogenicity and aciduricity as a competitive strategy to kill competitors.

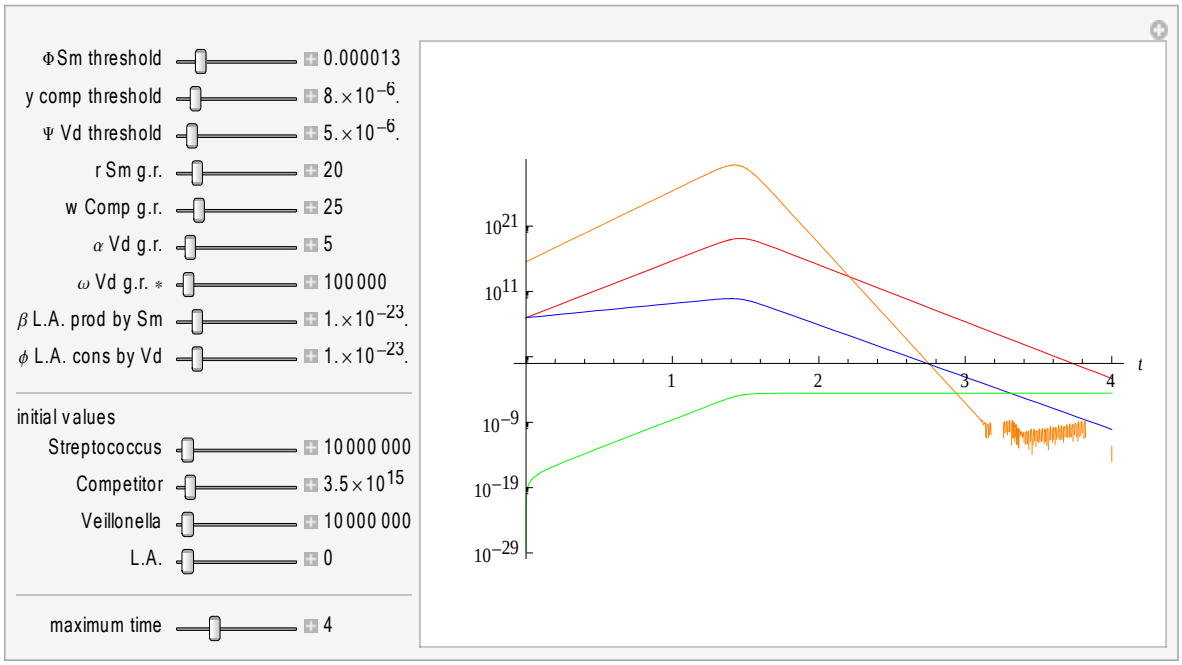


Figure 6.D Investigating the effect of the model output when the competitor massively outnumbers *S. mutans*. The populations of *S. mutans, V. dispar* and the competitor are shown in red, blue and yellow respectively. The concentration of lactic acid is shown in green.

Figure 6.D shows a similar run of the model to Figure 6.C, but where the competitor initially massively outnumbers *S. mutans* yet *S. mutans* can alter the environment making it too acidic for the competitor and thus after 2.3 days *S. mutans* outnumbers the competitor. It shows the competitive strategy of acidogenicity and aciduricity, producing and tolerating large quantities of acid, could be extremely effective against a competitor. This would not be able to happen if the competitor is metabolising glucose (in to something other than lactic acid) such that it runs out before *S. mutans* can metabolise enough of it into lactic acid to reach the critical threshold for the competitor. However glucose is just one of the many nutrients *S. mutans* needs and most species in the oral cavity do not use glucose as their primary energy source, yet many of these species would use nutrients that are useful to *S. mutans*, so these species would be in competition with *S. mutans* and these species would likely be susceptible to low pH.

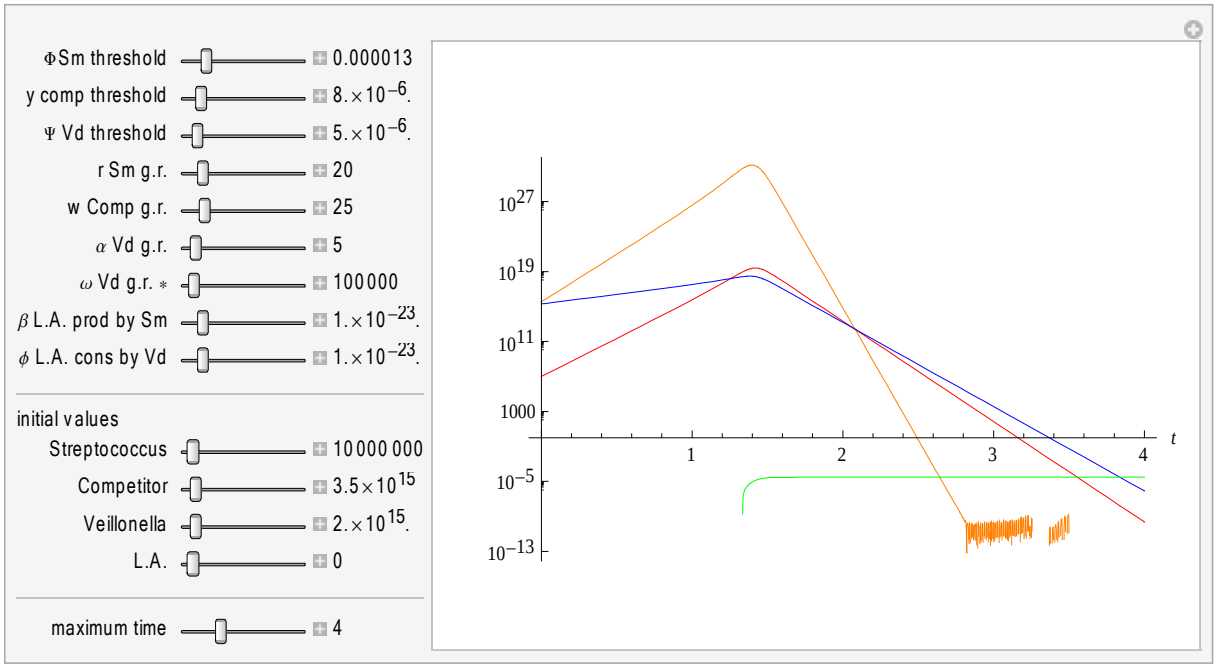


Figure 6.E Investigating the effect on model output when *V. dispar* greatly outnumbers *S. mutans*. This figure shows a scenario where *V. dispar* massively outnumbers *S. mutans* (8-fold difference). The populations of *S. mutans*, *V. dispar* and the competitor are shown in red, blue and yellow respectively. The concentration of lactic acid is shown in green.

Figure 6.E shows a run of the model where *V. dispar* massively outnumbers *S. mutans*. Despite *V. dispar* removing lactic acid from the system, the faster growth rate of *S. mutans* allows it to soon outnumber *V. dispar*, at which point there is a very rapid increase in lactic acid concentration (as can be seen by the steep gradient of the lactic acid concentration at time t=1.35), and the critical lactic acid threshold for *V. dispar* and the competitor are passed and reached (and slightly passed) for *S. mutans*. *V. dispar* metabolising lactic acid into weaker acids (treated as not being acidic at all in this model for simplicity) could help all species that are less aciduric than *S. mutans*, including competitors, although in this model this is not a strong effect as the lactic acid can be produced so rapidly.

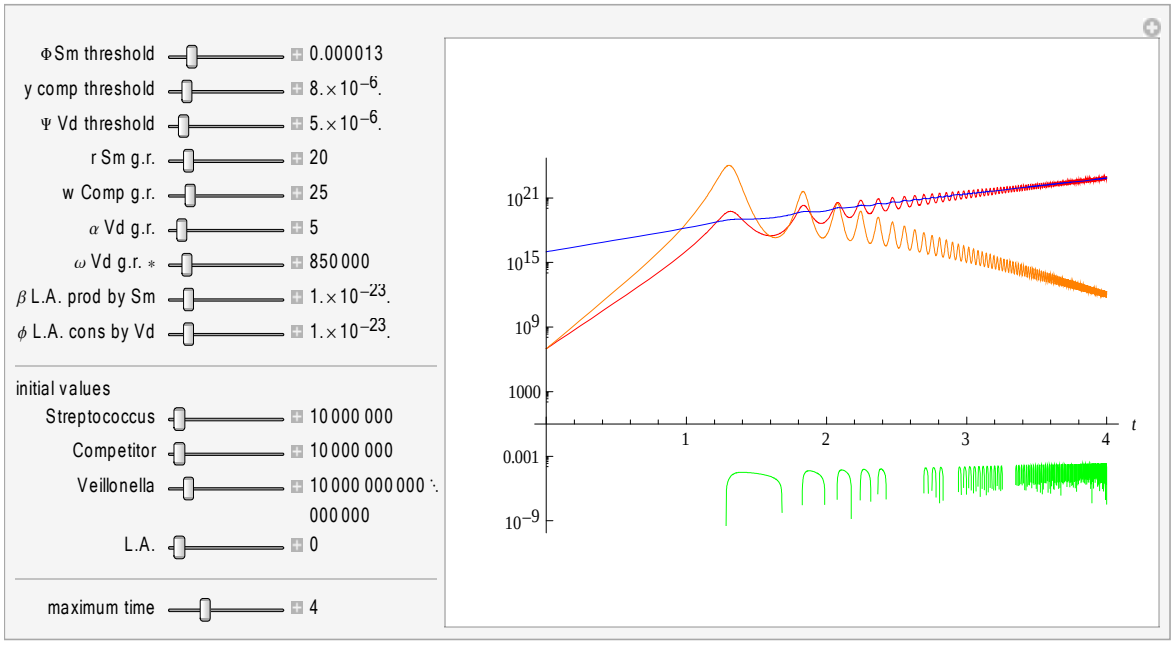


Figure 6.F The model output when *V. dispar* greatly outnumbers *S. mutans* and has a greatly increased growth rate in the presence of lactic acid. In this hypothetical situation, *V. dispar* initially massively outnumbers *S. mutans*, but it also has a greatly increased growth rate in the presence of lactic acid (ω). The populations of *S. mutans*, *V. dispar* and the competitor are shown in red, blue and yellow respectively. The concentration of lactic acid is shown in green.

A hypothetical system was tested where *V. dispar* had a large gain in growth rate in the presence of lactic acid, and where *V. dispar* greatly outnumbered *S. mutans* initially. This is shown in Figure 6.F. *V. dispar* grows exponentially. *S. mutans* grows exponentially and then oscillates around the population of *V. dispar*, but with a trend of growth. The competitor grows exponentially and then begins to oscillate but with a gradual trend of decline. The oscillations seen in the populations of *S. mutans* and the competitor are caused by oscillations in the lactic acid concentration shown in green. It shows an example where the two species do co-operate; they both increase in number despite large oscillations in lactic acid concentration, while at the same time they outcompete the competitor. This run was conducted to investigate the possibilities of control for this system, either through screening or modifying a bacterium to possess the qualities of the hypothetical strain of *V. dispar* used in this run of the model. It shows it could be possible for a species to persist even if the pH briefly drops below its critical threshold where growth ceases and the bacteria start to die, but it also shows the average pH over the period is low and would be harmful to enamel. Thus the potential for a holistic approach using a probiotic cooperative strain

that metabolises lactic acid is poor, as the average pH may still be at a level that is harmful to teeth.

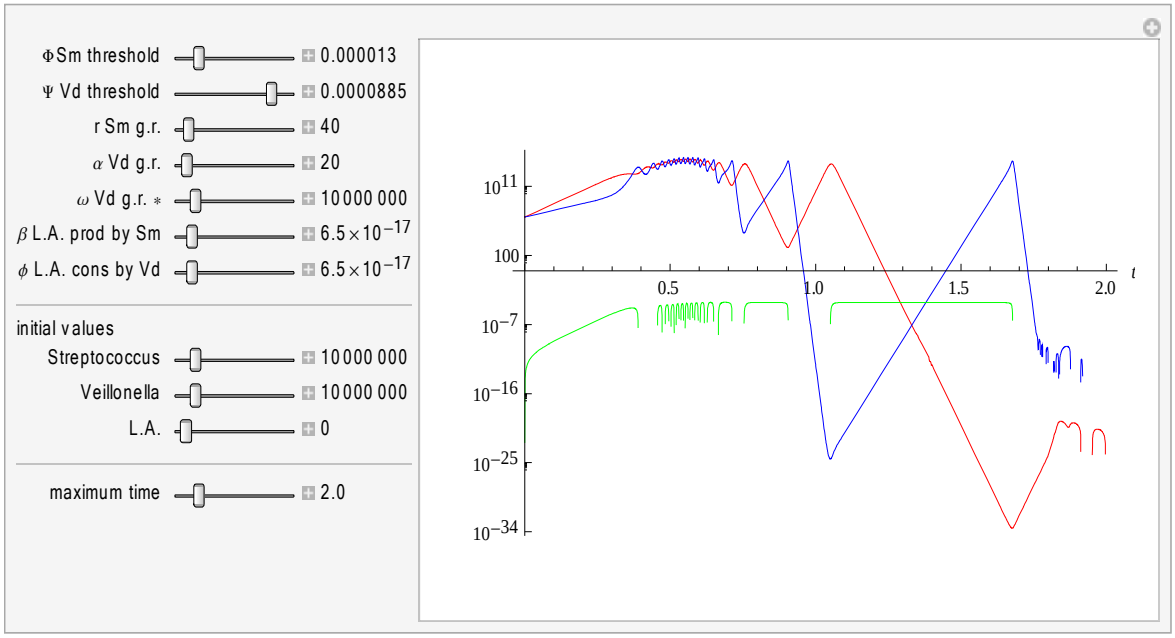


Figure 6.G Hypothetical situation showing oscillating nature of relationship if *V. dispar* were to have a higher tolerance to acidity than *S. mutans*

In this last scenario using this model, the effect of *V. dispar* being more aciduric than *S. mutans* was modelled. *V. dispar* also had a greatly increased growth rate in the presence of lactic acid. The populations of *S. mutans* and *V. dispar* are shown in red and blue respectively. The concentration of lactic acid is shown in green.

Another hypothetical situation was tested in a run of the model where *V. dispar* was more aciduric than *S. mutans* which is shown in Figure 6.G. In this scenario the two species are seen to have a trend of exponential growth and death but with the species oscillating around each other. The concentration had a trend of exponential growth before plateauing at around 10^{-5} but with sudden drops in concentration when *V. dispar* was consuming the acid much faster than *S. mutans* was producing it. As with Figure 6.I, this is an extreme example to demonstrate the predictive power of modelling. This is a case that does not exist, so could not be tested experimentally, but is of interest in understanding the system as it shows what could potentially happen if a genetic engineering approach was taken to controlling caries by modifying a symbiont to be more acid tolerant. The low values after t=1.0; are still positive but very low concentrations of each species. The acidity is at a point where it is harmful to teeth, so again this control approach is unlikely to work.

6.3.2 Competing Growth Processes

The incipient growth processes model separates the processes of growth and death, assigning two descriptive parameters to each and thus is better able to describe the pattern of the growth observed in the experimental chapter. It however has a very flexible model form and five parameters so the fact it can fit five data points means little. It does however show that separating growth and death and assigning two parameters to each can produce the characteristic shape found in the experimental results (see Figures 6.H and 6.I).

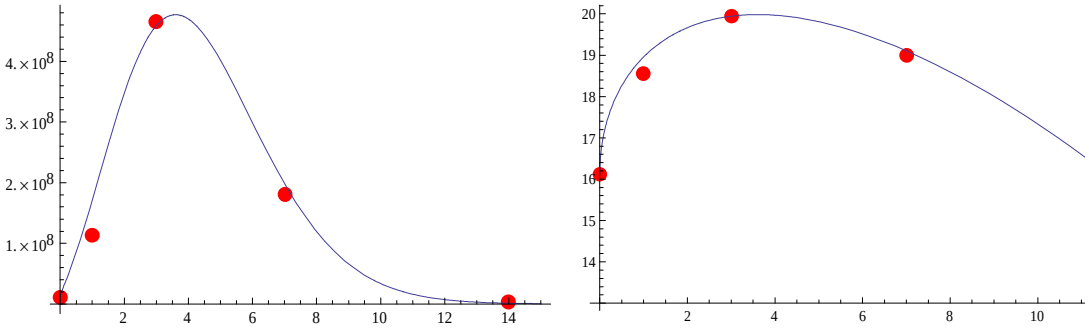


Figure 6.H Plot and logplot of incipient growth processes model with competing mechanisms model using base 2. Plot and logplot of model and representative coordinates using parameters: $y_0=10^7$, $t_{c1}=0.0435$, $m_1=0.489$; $t_{c2}=1.58$, $m_2=1.37$. Red dots are population sizes measured in the experiments conducted in Chapter 3.

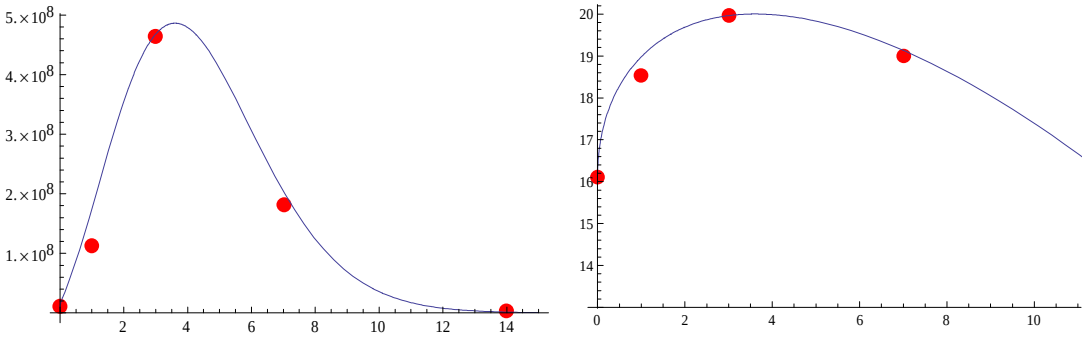


Figure 6.I Plot and logplot incipient growth processes model with competing mechanisms model using base e. Plot and logplot of model and representative coordinates using parameters: $y_0=10^7$, $t_{c1}=0.09$, $m_1=0.49$; $t_{c2}=2$, $m_2=1.35$. Red dots are population sizes measured in the experiments conducted in Chapter 3.

A problem with the form of this model is that while growth and death both had a rate parameter, the other parameter was a time parameter – essentially a switch point. Having this second parameter greatly improved the fit of the model but it would be good to calculate these parameters within the model using the underlying biological fundamentals. Only the model and representative data points for *S. mutans* are shown here as this model is solely of growth and decay and not of the interaction of the two species.

Figure 6.H and 6.I show that either base 2 or base e can be used (to reflect growth as a result of fission [base 2] or for mathematical convenience [base e]). Because five points were fitted to five parameters, goodness of fit tests (e.g. RMSE, Root Mean Square Error) to compare the two models (base 2 and e) were not done.

6.3.3 Summary of Results

This chapter employed mathematical modelling techniques to investigate the growth and decline of *S. mutans* and *V. dispar* biofilms.

- The initial model which used parameters that were set or measured in the experiments conducted reached the maximum peak for *S. mutans* and *V. dispar* after 0.009 days indicating the parameters and/or the model were not sufficient to recreate the experimental results accurately.
- Consequently the model was treated as dimensionless to investigate the dynamics of the system and the parameters were changed to better reflect the experimental results.
- Shortly after the lethal concentration of lactic acid was exceeded for *V. dispar* it was also reached for *S. mutans* because the concentration increased so rapidly.
- Results imply the species are indeed coupled around lactic acid and that this relationship is responsible for the pattern of growth and decline of both species.
- Model supports the new hypothesis that *S. mutans* employs a strategy of acidogenicity and aciduricity to outcompete other species and that this strategy could allow *S. mutans* to outcompete another species, even if it is outnumbered and the other species has a faster growth rate, as long as *S. mutans* is more aciduric than the other species.
- The rapid growth rate of *S. mutans* indicates it will rapidly produce sufficient lactic acid to kill *V. dispar* even if *V. dispar* massively outnumbers *S. mutans* initially.
- Two hypothetical scenarios, that could not be tested experimentally, were also used to show how useful modelling can be. Possible ways to genetically engineer *V. dispar* were evaluated, including making it more aciduric, but these strategies were shown to be ineffective in the reduction of caries.
- A different type of model was also tested to investigate ways of constructing a model that more closely approximates the experimental data.

6.4 Discussion

This chapter describes the approach taken to model the subset of the dental plaque biofilms investigated in the laboratory studies. A non-monotonic growth model was developed that reproduced the basic coupled growth curves observed in Chapter 3, indicating that lactic acid is likely to be the most significant factor in describing the growth of these two species, however the actual values output by the model were very different to those observed experimentally. Thus another model form was investigated to inform possible ways to improve the non-monotonic growth model.

6.4.1 The Interaction of *S. mutans* and *V. dispar*

The relative starting values matter little because of the exponential rate of growth. The population sizes reached are dependent upon the factors that limit growth. When *V. dispar* massively outnumbers *S. mutans*, the rapid growth of *S. mutans* allows *S. mutans* to produce sufficient lactic acid to cause ecological collapse. Acidification to lethal levels can occur very rapidly. This is similar to results found in experimental analysis of multi-species biofilms where glucose was pulsed into the system and the pH dropped below 5 within one hour of the pulse (Bradshaw et al. 2002).

V. dispar, by removing acid and raising the pH, helps all acid sensitive species in the community, although the model indicates this is not a strong effect. In a more complex community with many species taking up valuable space, using up limited nutrients and introducing more products in the biofilm (some of which would have a buffering effect) the growth of *S. mutans* would be limited and thus the production of lactic acid would be limited, potentially enabling *V. dispar* to metabolise lactic acid fast enough to balance the production of lactic acid by *S. mutans*, and thus maintain pH at a level that benefits many of the species in the community, but importantly not *S. mutans*. Consequently the very interaction that is described as potentially cooperative (Rogosa 1965, Mikx et al. 1972, van der Hoeven et al. 1978, McBride and van der Hoeven 1981, Mays *et al*. 1982, Hoshino and Sato 1986, Kara et al. 2006, Palmer et al. 2006, Chalmers et al. 2008) may be competitive.

6.4.2 The Addition of a Competitor

S. mutans can outcompete a competitor if it has sufficient glucose to metabolise into lactic acid to pass the critical pH threshold of the competitor. The model strongly supported the hypothesis that acidogenicity and aciduricity could be a very effective strategy to achieve ecological hegemony as it showed *S. mutans* could poison the environment for a competitor. This approach could be extended to investigate the dynamics of a case where the species are competing for a resource, with the obvious choice being glucose.

Kemp et al. (1983) modelled simple competition for a single energy source for *S. mutans* and *S. sanguinis* competing for glucose in batch and chemostat culture and identified factors other than competition for glucose were operating as well. They found the order of inoculation was important while the inoculum size was not, which suggests both species were producing bacteriocins and the first species to colonise the environment would poison it for the other one. Both *S. mutans* and *S. sanguinis* metabolise glucose into lactic acid and other acids but *S. mutans* has a competitive advantage at lower pH (Bowden and Hamilton 1987). The main competitors of *S. mutans* are other streptococcal species, and these species are acidogenic and aciduric as well, although not to the same extent as *S. mutans*. Thus *S. mutans*, as shown in the models, could outcompete them by producing sufficient lactic acid to kill them but it is likely the main competitive interaction between Streptococcal species will be through bacteriocins and H_2O_2 (Qi and Kreth 2010). Non-Streptococcal species which will mostly be susceptible to low pH may compete with *S. mutans* for nutrients.

6.4.3 Identifying Ways to Beneficially Modulate the System

The combination of acidogenicity and aciduricity appears to be a highly effective survival strategy for *S. mutans*, so there is little chance that modifying a strain of *S. mutans* to be ‘more fit’ but less harmful, such that it could outcompete the wild-type strain of *S. mutans* but would produce less acid, is a possible treatment strategy.

Probiotic bacteria and prebiotics are a major industry for gastrointestinal health, yet until recently all control methods for oral health have focused on removing or killing all oral bacteria. Teeth which function to cut and pulverise food are essentially inert and the bacteria that are there are thought to not be useful, and mechanical removal is a very effective way to remove bacteria but it is process-intensive. However some of the bacteria in plaque may be useful or could be useful. Ways to potentially modify this community were investigated with the idea of developing a probiotic bacterium that would reduce the occurrence of caries. As modifying *S. mutans* is unlikely to work as a strategy to prevent caries as discussed earlier, the focus moves away from *S. mutans* and on to *V. dispar*. However the initial modelling attempts show little promise for screening for or genetically modifying a strain that would metabolise the acid in to less strong acid products, as *S. mutans* still appeared to be capable of lowering the average pH to harmful levels.

As this modelling approach did not model the effects of space it may yet be possible to mechanically remove all the bacteria and then inoculate in high numbers with symbionts which could competitively exclude *S. mutans* and possibly metabolise lactic acid or produce buffering compounds. A novel probiotic approach has recently been patented where *Lactobacillus paracasei* which aggregates with *S. mutans* is topically applied to the teeth and it clumps together with *S. mutans* bacteria facilitating their removal (Lang et al. 2010, Tanzer et al. 2010).

6.4.4 The Model

6.4.4.1 Assumptions of model

The model only looks at the single process of the production and removal of lactic acid and the effects lactic acid has on the two species. It assumes production and consumption of lactic acid are dependent upon the number of bacteria of each species respectively which is not correct as both will reduce as the pH decreases. It treats lactic acid as a proxy for [H+] which is a simplification as while hydrogen ions are causing damage, undissociated lactic acid can cross the membrane barrier and cause damage or interfere with cellular processes internally (Lambert and Stratford 1999, Janssen et al. 2007). The growth rate is reflected in the death rate as they are multiplied together using the form of this equation. This is not unreasonable as many

species that grow faster also die faster, as proportionally more of their resources are put in to growth rather than survival. Diffusion and spatial information are not included in the model, which greatly simplifies the process of modelling this system but limits the nature of the interactions and environmental changes that can occur. The limited spatial structure of the biofilms observed using the confocal microscope makes this more valid an assumption, but the dense biofilms that grow may develop strong nutrient and pH gradients in the axis perpendicular to the nitrocellulose filter.

6.4.4.2 Problems with the model

The model reproduces the shapes of growth of, and the interactions between, these two species but it does not reproduce the values measured in the experiments. Currently lactic acid concentration can become negative and form a negative multiplier in the growth rate (if there are many more *V. dispar* than *S. mutans*) producing sudden rapid declines in population numbers. Finally, aside from the lower critical pH values where growth ceases (biofilms are more resistant to pH, Kubota et al. 2008), there is nothing to make this a specifically biofilm rather than planktonic model, which is not inherently bad as general models are good, but the focus of this research has been on biofilm growth.

6.4.4.3 Ways to improve the model

There are two main ways to improve the model; improve the form of the model so it has a better fit to measured data, and include a second process, the consumption and possible depletion of one or more resources. The form of the incipient growth equation can be used to inform the changes to make to the non-monotonic growth model. One example of this will be to separate growth and death in the form of the model. Also the parameter for time could have its units changed from days to hours, specifically with regard to improving the fit of the growth rate parameters. The second phase will be to include an additional process taking into account limited nutrients. Lactic acid is the only nutrient included in the model (*S. mutans* has no nutrient information in the model) and it is the only one that can be depleted; there is no pool of essential nutrients that can be depleted. The main way to model substrate

utilisation is to employ the method developed by Monod. Jacques Monod (who shared the Nobel Prize in Physiology/Medicine for the discovery of mRNA) developed the Monod function which relates the concentration of a limiting nutrient to a population's growth rate (Lobry et al. 1992).

$$\mu = \hat{\mu} \frac{S}{K_S + S}$$

In this equation μ represents the specific growth rate coefficient, $\hat{\mu}$ the maximum specific growth rate coefficient, S the concentration of the limiting nutrient, and K_S the half saturation coefficient. This equation could be integrated in to the model to model the depletion of glucose and its conversion to lactic acid. Once the model is accurately reproducing the experimental results it can then be used to make testable hypotheses.

Logistic models are used to model food safety, primarily to identify K and the rate of growth of harmful bacteria but little work has been done towards the death of populations at low pH (with the notable example and review of the few previous attempts of Janssen et al. 2006). Lactic acid has been used as a means of preserving food for millennia (e.g. sauerkraut where lactic acid bacteria ferment the sugars in cabbage producing an environment unfavourable to most other bacteria and thus preserving the food). Logistic models have been developed to determine K in dual species bacterial cultures containing a species of lactic acid bacteria and a species of harmful bacteria (Vereecken et al. 2003). This system is very similar to the system investigated in this study however with the pathogenic and symbiotic bacteria having switched. When this model is working it should be of use to the food industry in modelling this interaction (including the death phase).

6.4.5 Comparison with Other Models and Experiments

6.4.5.1 Comparison with other models and experiments – co-operative behaviours

Many newer models are far more complex yet this simple model captures the basic nature of the relationship. This study was able to describe the pattern of growth

without modelling spatial components, yet the next two studies explicitly model biofilm development in three dimensions to investigate purportedly cooperative behaviours.

A biofilm modelling study by Xavier and Foster (2007) investigated the hypothesis that production of extracellular polymeric substances is a cooperative act requiring a high level of cooperation, only to reject this hypothesis having shown that there is a strong evolutionary advantage to producing these polymers as they push later generations into more favourable oxygen conditions while suffocating non-polymer producing bacteria, in much the same way plants in a forest grow towards light and the canopy limits the light getting through to plants below. Their study by necessity explicitly calculated diffusion-reaction effects and the resulting solute gradients, but it, like this study, showed a purportedly cooperative behaviour is actually an evolutionarily advantageous competitive behaviour.

Confocal microscopy has revealed elaborate mushroom caps in *Pseudomonas aeruginosa* biofilms (Klausen et al. 2006) and modelling studies have attempted to describe how these can form (Picioreanu et al. 2007). These were initially thought to be cooperative structures but it is possible they form by individual bacteria maximising their own access to available substrates, another example of a proposed cooperative behaviour that is better explained by invoking fitness advantages to the individual bacterium (Klausen et al. 2006, Picioreanu et al. 2007).

However modelling has provided some evidence for the existence of cooperative behaviours in biofilms by showing that altruistic bacteria can outcompete cheaters (organisms that take advantage of a cooperative behaviour without contributing themselves) if they gain an advantage from their cooperative behaviour that allows them to disperse more, as the dispersed bacterium can form a new colony that is free from cheaters (Kreft and Bonhoeffer 2005).

6.4.5.2 Comparison with other models and experiments – growth and acidity

Many studies using modelling and experimental approaches have found early induction of the stationary phase in co-culture studies with one species producing lactic

acid (Janssen et al. 2006, Mellefont et al. 2008, and Vereecken et al. 2003) in agreement with the findings of this research.

In contrast to this study, inoculation sequence and inoculation ratio have been found to predict total viable counts after a period of growth in some experiments (Donoghue et al. 1983, Mellefont et al. 2008) while in other experiments they do not (Mellefont et al. 2008) which is in agreement with this study. Kemp et al. (1983) found inoculation ratio was not important which is in agreement with the results of this study. It appears the interaction that occurs is pairing specific, where if the first or more prolific species produces inhibitory compounds it can maintain dominance. This occurs even if the other species also produces inhibitory compounds, but if the first species does not produce inhibitory compounds, and the other species does, the first species will loose dominance.

This model is significant as it models growth and decline for two interrelated species and to the author's knowledge it is a novel functional form. The model's results support the hypothesis that *S. mutans* and *V. dispar* are not in a commensal relationship as *S. mutans'* acidogenesis and aciduricity is a strategy to gain ecological dominance, and the removal of lactic acid by *V. dispar* interferes with this strategy. Finally, the hypothetical scenarios tested indicated limited potential for the effectiveness of a lactic acid metabolising probiotic bacterium. However this model only looks at the single process of the production and removal of lactic acid, yet there are many determinants that increase and decrease the pH of the microenvironments within the oral cavity (Wilson 2005).

7 Final Discussion: Implications for the Management of *S. mutans* and for the Understanding of Species Interactions in Bacterial Biofilms

7.1 Main Conclusions

This research was conducted with one of the aims being to investigate how cooperative behaviour evolved and can be maintained, but it came to pass that the system hypothesised to be cooperative is actually competitive. Biofilms are communities of bacteria but this does not mean they are cooperative. Community members still act selfishly and many features of biofilms and behaviours of bacteria are either unselected or are actually competitive. Bacteria have to bind to surfaces in the oral cavity, as if they attempt to live planktonically for any length of time they will be washed away in the saliva and swallowed. Once bacteria have bound they need to stay bound, so biofilms can arise without any cooperation but solely because it is evolutionarily advantageous for bacteria to attach and stay attached to anything they can.

The main conclusion of this thesis is that lactic acid is not a waste product but rather a chemical warfare agent. This shifts the focus of potential ways to beneficially modulate this community away from cooperative behaviours within dental plaque and on to competitive interactions. Dental plaque changes from being a relatively benign community living on our teeth, to being the cause of disease, as a result of a change in community composition and plaque metabolism when there is an abundant supply of dietary carbohydrates that can be fermented into lactic acid. Marsh proposed the ecological plaque hypothesis in 1991 stating that a change in environmental factor or factors can trigger a change in community composition that may predispose a site to disease (Marsh 1991). This research has indicated that the change in community composition is probably a direct result of the production of lactic acid. This implies that the supply of carbohydrates is the trigger for this change, however given the normal human diet this would happen very frequently so it is likely to be more complex than this. Many papers report on the requirement of a mature biofilm before caries can form (e.g. Takahashi and Nyvad 2007, Kolenbrander et al. 2010). This may not be the case but if it is, why this is required is important in understanding this system. *S. mutans* can attach directly to the tooth pellicle so it is not dependent upon the early colonisers for attachment, but it does have a relatively small genome (but with many transport mechanisms) so it needs to

acquire rather than build many of its essential nutrients. It is possible *S. mutans* requires the community of other species to be present in the biofilm so it can harvest their nutrients once it starts killing them with acid.

Becker et al. (2002) found healthy subjects had significantly higher numbers of *S. sanguinis* (and *S. gordonii* was higher but not significantly so) than subjects with caries, implying that the presence of these species can prevent *S. mutans* (and other highly acidogenic species) taking control of the ecosystem, and thus prevent the development of caries. *S. mutans* competes with *S. sanguinis* and an inverse correlation is observed in their presence in plaque, including around carious lesions (Loesche et al. 1975, Mikx et al. 1976, Caufield et al. 2000, Becker et al. 2002, Kreth et al. 2005). Whether *S. sanguinis* is not present in the plaque around caries because *S. mutans* has killed them all, or whether the presence of *S. sanguinis* can prevent *S. mutans* gaining ecological hegemony and thus cause caries, remains to be determined. *S. sanguinis* and *S. gordonii* produce H_2O_2 which inhibits the growth of *S. mutans* (Kreth et al. 2005). *S. mutans* can reduce H_2O_2 using AhpC but *S. sanguinis* and *S. gordonii* are more tolerant and can produce levels harmful to *S. mutans* that they themselves can sustain (Wu et al. 2010). This is a similar but reversed relationship to that centred on lactic acid, with all three streptococcal species being tolerant but *S. mutans* being more tolerant.

The requirement for plaque to form an established community before the onset of caries may simply be due to the buffering effect of saliva, as in established plaque there is relatively less of it and it can penetrate the biofilm less, or it may be because the proportion of non-mutans-streptococci which are early colonisers decreases in the ageing biofilm and their ability to inhibit *S. mutans* growth decreases. The stability of community composition in plaque, termed microbial homeostasis, is due to a delicate balance of competitive and cooperative interactions (Marsh 1994), and probably more of the latter. It is clear that if *S. mutans* is given the opportunity, it will disrupt the community and drive the system towards disease, as it gains a competitive advantage from doing so. Therefore it is the species in the community that can limit the growth of *S. mutans* that probably determine whether homeostasis can be disrupted. These species are probably the key to the prevention of disease (in combination with good oral hygiene and limiting the supply of carbohydrates to plaque). In the simple system studied here, *V. dispar* could not prevent the rapid

accumulation of lactic acid that resulted in ecological collapse, so it appears that antagonistic interactions directed towards *S. mutans* may hold the balance. Keeping the system simple and looking at just two species may have led to a more rapid ecological collapse as many of the interactions that would have slowed the development of *S. mutans* were not present.

In the introduction of this thesis, Figure 1.Q shows some nutritional interactions that occur between organisms in the oral cavity. The coupling of *S. mutans* and *V. dispar* is just a small subset of that figure, which itself is just a subset of all the nutritional interactions that actually occur. Given how complex the coupling of *S. mutans* and *V. dispar* around lactic acid is, the system as a whole will be extremely complex. It is not sufficient to assume two species will cooperate because they are coupled around a metabolic pathway. Another example of nature dividing a metabolic pathway into different pieces each processed by different species of bacteria, that is often cited as being cooperative when in fact it arose through competitive interactions, is the metabolic separation and stratification of ammonium oxidation and nitrate oxidation in wastewater treatment reactors which occurs because a bacterium that only oxidises the ammonia grows faster than one that oxidises both (Nadell et al. 2009). While bacteria can be more rigorously studied, scientists have been less rigorous in early descriptions of cooperative behaviour in bacteria, often stating it occurs without any evidence. Bacteria are cooperating some of the time (e.g. the production of iron scavenging siderophores, West and Buckling 2003) but their cooperation needs to be more closely tested. To understand this system it is imperative to study the social interactions of and evolutionary forces acting on the bacteria.

Ecological balance is the normal state of dental plaque (unless it is mechanically removed by brushing and flossing or killed with mouthwash). Dental plaque is very complex with many species, nutrients, metabolites, host effects and shear forces acting on it and most of the complexity of the biofilm will buffer against perturbations. Many species all acting in their own self-interest, that have evolved to require slightly different nutrients, will keep each other in balance, through density effects, where if a certain species increases in population, the nutrients that species requires will decrease proportionately, resulting in a different species, with different nutrient requirements growing faster and thus increasing in population in a continuing cycle

of minor fluctuations in relative population. Additionally these species are in competition with each other, and produce compounds toxic to their competitors, thus keeping the numbers of any individual species down (Kreth et al. 2008). In keeping with the complex and intricate dynamics of this ecosystem, *S. mutans* may at times be of benefit to us as hosts, as CSP inhibits the yeast-mycelium transition and thus *S. mutans* can prevent thrush (Jarosz et al. 2009, Joyner et al. 2010), a common oral disease in those who are immunocompromised.

The pH of the oral environment often drops to approximately 5.0 after pulses of carbohydrates but it normally returns rapidly to a more neutral pH. This occurs because the production of lactic acid ceases when the carbohydrate supply is exhausted, carbohydrates are washed away by saliva, acid is washed away by saliva, acid is buffered by saliva and metabolites produced by plaque bacteria, alkali are produced by plaque bacteria, lactic acid is metabolised by plaque bacteria and the metabolism of acidogenic bacteria is decreased due to low pH (Burne and Marquis 2000, Wilson 2005). When pH does not return to neutral, the composition of the plaque changes and caries can form.

7.2 Future Work – Direct Succession of this Work

As this study demonstrates the complex nature of some of the bacterial interactions in the oral biofilm, it would be interesting to evaluate whether the often cited cooperative bacterial interactions are actually cooperative. Further studies could be done that investigate competence as a nutritional strategy. This study demonstrated the advantages to using an enrichment strategy when extracting RNA from dual species biofilms, but this method could be improved further to determine optimal guanidium thiocyanate concentrations to differentially enrich for either Gram-negative or Gram-positive species. The 22 genes of unknown function that were upregulated in dual species biofilms are worth investigating further to identify if they are specifically interaction genes of novel function. Multi-species, multi-nutrient, multi-dimensional models exist that allow for the modelling of nutrient intermediates that are produced and consumed at different locations within the biofilm (Kreft et al. 1998, 2001; Picioreanu et al. 2004). One of these could be modified so that it behaves in a similar way to the biofilms that have been grown in the laboratory studies. The model would be based on diffusion-reaction mass balances of glucose and lactic acid, coupled with

microbial growth as characterised in the laboratory studies. The metabolism and growth of individual bacteria could be modelled in three dimensions to recreate the flux of lactate and multidirectional pH gradients. Relevant genetic factors identified in the transcriptomic analysis could be included as well. However this may not provide that much more information and predictive power than the current model. The current model could be expanded to include substrate utilisation as described in section 6.4.4.3. Additionally the gene expression data could be incorporated in to a flux balance model of *S. mutans* metabolism. *S. mutans* can make formate which converts to formic acid in solution. Formic acid is more acidic than lactic acid so it would be interesting to determine why *S. mutans* is not producing formic acid to achieve ecological hegemony as producing it might lower the pH past the critical levels for other species faster. The results of the experimental and modelling studies in this thesis indicate that modulating the plaque community is likely to be best achieved using species that compete with, rather than cooperate with, *S. mutans*. Non-mutans-streptococci, particularly *S. sanguinis* and *S. gordonii* may serve as suitable probiotic bacteria to prevent ecological collapse due to the production of acid by *S. mutans.* The function of *S. gordonii* and *S. sanguinis* as probiotic bacteria could be tested in vivo by spreading cultures of these species on to professionally cleaned rat teeth, and using controls determine if these putative probiotics can inhibit *S. mutans* growth and thus prevent the development of carious lesions when the rats are fed a diet rich in fermentable carbohydrates.

7.3 Future Work - Development of Field

It is important to establish whether, and if so why, a complex community of plaque is required for the development of caries. Approaches that subvert the *S. mutans* signalling pathways could be developed that either trigger or inhibit the signal (Burne et al. 2009) which other streptococci do already (interference with *S. mutans* CSP signalling). For example the *S. mutans* carbon catabolite repression signalling pathway could be tricked to prevent *S. mutans* converting pyruvate into lactate, or the acid stress signalling pathways could be triggered earlier. Brown et al. (2009) have proposed the use of Trojan horse strategies to treat bacterial disease through employing our understanding of competitive and cooperative interactions of bacteria to introduce less virulent strains or beneficial genes into communities. It is unlikely that *S. mutans* could be modified to produce less acid as it gains such a

competitive advantage due to the production of lactic acid but it could be modified to be less virulent. An example of this would be to remove *S. mutans'* genes for bacteriocins, effectively producing cheats that benefit from the production of bacteriocins by wild-type *S. mutans* but don't share the cost of production and thus grow faster, enabling them to increase in relative proportion in the biofilm while decreasing total bacteriocin production and thus possibly enabling other streptococcal species to limit the growth of all *S. mutans*. Additionally these competitors could have advantageous genes added to their genomes to assist them in limiting the growth of *S. mutans*.

As caries is a polymicrobial disease, any methods that do not solely target *S. mutans* will prevent a similar acidogenic species from filling the niche vacated by *S. mutans*. *V. dispar* was killed by the pH drop caused by *S. mutans* but other veillonellae are more aciduric and may survive the initial acidification of the environment long enough to raise pH to levels that are not harmful to teeth, but these veillonellae are behaving selfishly towards *S. mutans* and should be viewed as competitors as they get the benefit of food while *S. mutans* loses its competitive advantage against less aciduric species.

As more oral bacteria genomes get sequenced, and as microarray chips are increasingly easy to produce, whole genome transcriptomic studies of multiple species will be employed that improve our understanding of bacterial interactions but also of dental plaque. Finally, the human microbiome project (http://nihroadmap.nih.gov/hmp/) which is characterising the entire human microbiota will determine whether humans differ in their microbiomes and whether these differences correlate with disease or health. The first curated human microbiome is of the oral microbiome (www.homd.org, Dewhirst et al. 2010). Understanding how bacteria interact with each other will be immensely valuable in processing the riches of data that the human microbiome projects are starting to generate.

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9 Appendices

9.1 Appendix 1 – Microarray Application

National Institute of Dental and Craniofacial Diseases Oral Microbe Microarray Initiative (NOMMI)

Microarray Application

For the attention of:
Pamela McInnes, D.D.S., M.Sc. (Dent.)
NIDCR Microarray Initiative
Building 45; Room 4AN-12B
Bethesda, MD 20892-6402

Proposal Title:
Systems Ecology of Dental Plaque Biofilm

Summary:
We propose to use DNA microarrays to identify how competitive and cooperative interactions in a mixed species biofilm alter the global transcriptome of the primary etiological agent of dental caries.

Targeted Organism:
Streptococcus mutans UA 159

Principal Investigator:
Professor Michael Wilson
(Eastman Dental Institute, University College London)

Other Applicants:
Professor Robert M Seymour (Mathematics and CoMPLEX, University College London)
Professor Brian Henderson (Eastman Dental Institute, University College London)
Mr Will Koning (Eastman Dental Institute and CoMPLEX, University College London)

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Research Plan

Summary:
We propose to use DNA microarrays to identify how competitive and cooperative interactions in a mixed species biofilm alter the global transcriptome of the primary etiological agent of dental caries.

Specific Aims:
We aim to investigate how changing the community composition in the dental plaque biofilm changes the genes expressed by *S. mutans*, the primary etiological agent of caries.
We aim to use this information, in combination with proteomic, metabolic and microscopic methodology, to develop a quantitative model of the development and maintenance of the dental plaque biofilm.

Significance of Research Question:
Bacterial diseases are still major causes of mortality and morbidity worldwide. There is growing realization that we cannot deal with bacterial diseases using blunt instruments such as antibiotics and that to develop effective control of such diseases demands a complete understanding of the systems ecology of bacteria as it pertains to human disease. Bacteria generally live in the form of biofilms and we know very little about what signals control biofilm formation and maintenance. This study aims to use a multi-pronged approach to unravel the processes occurring in the formation and maintenance of the dental plaque biofilm, a complex ecological system. Understanding the systems ecology of this biofilm will help scientists to develop strategies to control the adverse effects of biofilms in general and in particular reduce the incidence of dental caries which are one of the most prevalent microbially-induced diseases of humans. We aim to develop a mathematical model of the oral biofilm community that incorporates altruism and competition between species.

Potential Impact on Field:
Understanding the systems ecology of human biofilms would provide basic information on how to control these systems and may allow for the development of novel non-antibiotic means of controlling bacterial disease or ways to beneficially modulate the community composition of biofilms. Understanding the systems ecology of the interactions of bacteria within multi-species biofilms should be of generic value to the study of bacterial diseases, no matter the causative agent.

Experimental Design to be Used:
We are currently quantifying and visualizing the dental plaque biofilm *in vitro* using total viable counts, live/dead counts and confocal microscopy. We are also investigating the proteomics and metabolomics of this community to understand the interactions occurring within and between species. We would like to extend this to investigate changes occurring in the global transcriptome of *S. mutans UA 159* as we vary the composition of the community and sample the biofilm over its formation, growth and maturation. Currently we are investigating *S. mutans* coexistence with a competitor *Streptococcus sanguinis*, a cooperator *Veillonella* dispar, and a major coaggregatory organism *Fusobacterium nucleatum*. As *S. sanguinis* is likely to hybridise with the *S. mutans* microarray we aim to exclude it and investigate *S. mutans* biofilms, *S. mutans* and *V. dispar* biofilms, *S. mutans* and *F. nucleatum* biofilms and *S. mutans*, *V. dispar* and *F. nucleatum* biofilms. We shall also assay *V. dispar* and *F. nucleatum* biofilms as a negative control for the hybridization. We aim to grow these five types of biofilms, anaerobically on nitrocellulose filters on Tryptone Soya agar supplemented with D-glucose, and sample at one, three and six days. We aim to sample mRNA using RNAprotect (Qiagen), TRIzol reagents (Life Technologies), bead beating in a reciprocal shaker and RNeasy (Qiagen). We will employ the standard established methods for labeling (using Cy3 and Cy5) and

hybridization of mRNA to the microarrays as outlined on the PFGRC website. As we will be extracting mRNA from mixed-species biofilms with varying compositions, we will standardize the amount of *S. mutans* mRNA hybridized based on the total viable counts done of the biofilms. We will use a repeated-dye-swap common-reference design comparing three biological replicates against the common reference of a *S. mutans* biofilm grown alone for one-day. With the repeated dye-swap and three biological replicates this will require six microarrays for each comparison. We aim to investigate the four biofilms involving *S. mutans* mentioned above at one, three and six days of growth, which will require 72 microarray slides (6x4x3). We also wish to compare the microarray against the *V. dispar* and *F. nucleatum* biofilms to test for non-specific hybridization which will require a further six microarray slides (one biological replicate with dye-swapping for each of the three growth periods). Thus we request a total of 78 microarray slides. We will collect the data on one of the UCL microarray readers, scanning first in the Cy5 channel as it is more sensitive to photodegradation than Cy3. We shall use the data analysis tools, TIGR Spotfinder, MeV-MeV, SAOPMD and the data management system MyOPMD. We will provide full information of all experimental conditions and data ensuring compliance with MIAME standards. The biological and technical replication employed by our design will allow strict statistical analysis of the patterns of gene expression in these systems. We shall then combine *S. mutans* whole genome transcriptome data with our other data to build a model of the systems ecology of the dental plaque biofilm.

Slides Requested:
We would like 78 high quality microarray slides plus a few low quality slides to validate our technique.

Resources and Environment

Key Personnel

This application brings together a microbiologist, a mathematician, a cell biologist and a geneticist to explore the systems biology of the formation and maintenance of oral biofilms.

Principal Investigator:
Professor Michael Wilson (Eastman Dental Institute, University College London) has his track record outlined in the previous Biographical Sketch. In short, he initially trained as a chemist before becoming a microbiologist whose major interest is oral microbiology. He has been working on biofilms since 1988 and has published more than 50 papers and reviews on biofilms, has edited two books on the subject and is the founder and Editor of the journal *Biofilms*. Professor Wilson shall oversee the entire project.

Other Applicants:
Professor Robert M Seymour (Mathematics and CoMPLEX, University College London) is Professor of Biomathematics at University College London and a founder member and co-organiser of CoMPLEX, (Centre for Mathematics and Physics in the Life Sciences and Experimental Biology) the multidisciplinary centre at UCL for promoting systems biology research. Professor Seymour will oversee the microarray analysis and the systems ecology modeling.

Professor Brian Henderson (Eastman Dental Institute, University College London) is a cell biologist who has focused on the cellular and molecular microbiology of cell surface and secreted proteins of oral pathogens. Professor Henderson will oversee the proteomics and metabolomics research that ties in with the transcriptomics research proposed in this application.

Mr Will Koning (Eastman Dental Institute and CoMPLEX, University College London) is a molecular geneticist with over 10 years technical experience. He will grow the biofilms and process the microarrays.

Facilities

Biofilms will be grown at the Eastman Dental Institute which was awarded the highest possible grade in its last ‘Research Assessment Exercise’ and a Queen’s Award for Excellence. Microarrays will be processed at the University College London Microarray Centre. Analysis will be conducted under the interdisciplinary umbrella of CoMPLEX, the United Kingdom’s leading interdisciplinary Systems Biology research centre.

The combination of the extensive knowledge base of the applicants and the advanced facilities within University College London ensure the proposed research has a high probability of success and will make excellent use of the microarray slides requested.

Data Agreement

We agree to release the data in a timely manner in a publicly available database.

We also agree to adhere to MIAME standards for the recording and reporting of microarray-based gene expression data.

9.2 Appendix 2 – Development of Methodology

The preliminary experiments effectively developed and refined the methods of growing and assaying biofilms.

9.2.1 Development of Methodology - Biofilm Growth Model

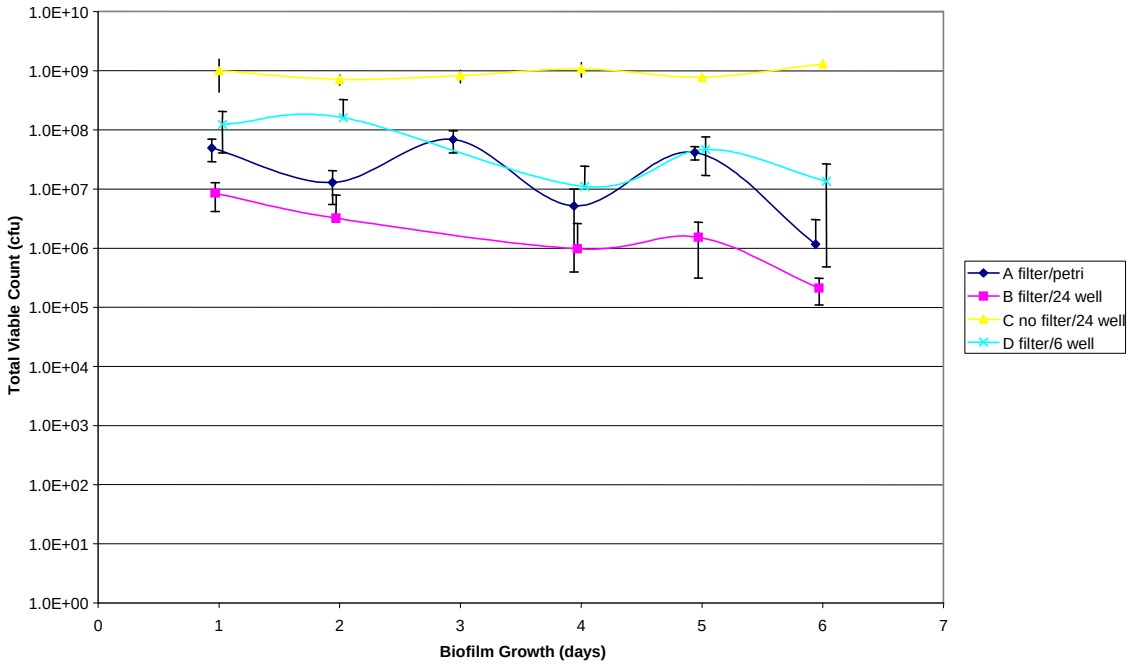


Figure 9.A. Total viable counts of *S. mutans* using four growing methods. The lines connecting the points are added to aid in distinguishing between the treatments and to help assess their variability and are not intended to imply the true trend in total viable counts for the treatments over time.

Four different growing methods were investigated to find a method that would reduce biological and sampling variation. Growing bacterial biofilms on a filter placed in a well of a 6-well tissue culture plate (Method D) proved to be a suitable method; as biofilm growth was similar between replicates and did not oscillate between days, samples were independent (chemicals could diffuse between replicates on filters placed on agar on Petri dishes) and the method consistently grew the second largest amount of bacteria (see Figure 9.A). Many more bacteria grew when grown directly on agar and the growth was similar between samples (day and repli-

cate) but this method would be unsuitable as removing the agar and recovering the bacteria was process intensive.

9.2.2 Development of Methodology - Growth and Screening Protocol

9.2.2.1 Removing bacteria from nitrocellulose filters and disrupting aggregations of bacteria

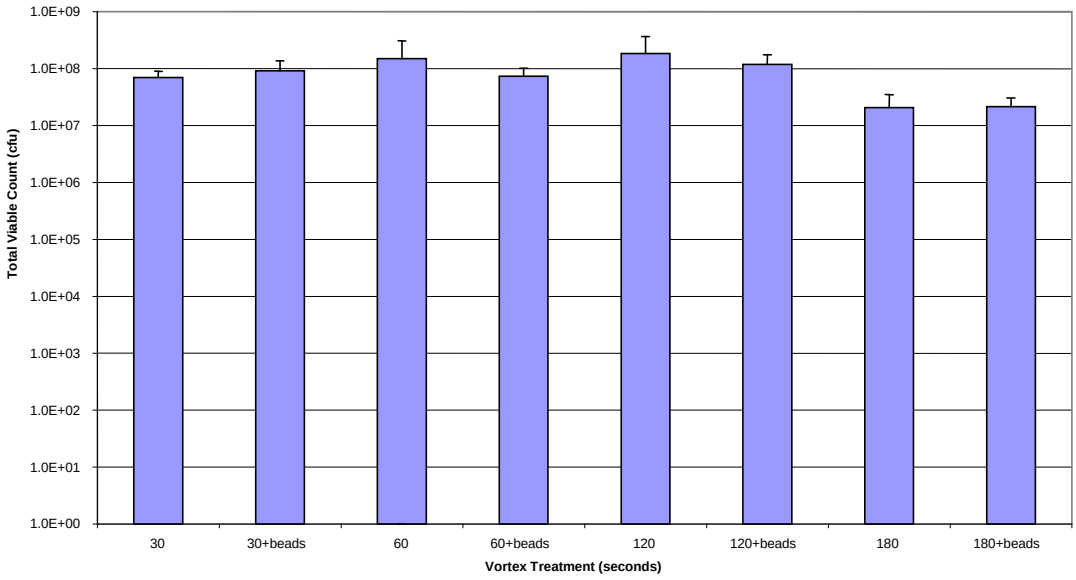


Figure 9.B. Different methods for the removal and disruption of *S. mutans* biofilms. Graph showing the total viable counts of eight different methods of biofilm removal from filters and disruption of aggregations. The methods used different lengths of vortexing with and without beads.

Different methods for the removal and disruption of *S. mutans* biofilms were trialled. The total viable counts of eight different methods, using different lengths of vortexing with and without beads, are shown in Figure 9.B. The methods differ in the total viable counts produced (one-way ANOVA, d.f.=7, F=2.846, P=0.014) but no one treatment produced significantly higher total viable counts than all the others (Dunnett's T3 post hoc tests for unequal variances) and the only significant differences between treatments showed that vortexing for 180 seconds, with or without beads, was significantly poorer than vortexing for 30 seconds without beads [P=0.008 for both 180 seconds and 180s with beads] and 60 seconds with beads [P=0.044 and P=0.048 for 180 seconds and 180 seconds with beads respectively]), so vortexing for two minutes without beads was chosen for subsequent analyses as it recovered the most viable bacteria on average (although not significantly; P values ranging from

0.515 to 1 for the comparisons against different treatment means) and it is a method used in the literature (Merritt et al. 2005a).

9.2.2.2 Identifying sampling regime, inter-experiment variability and trialling sampling methods

These experiments investigated biofilm growth across a range of time periods to identify suitable sampling points that represent the growth, maintenance and decline of *S. mutans* biofilms. Because of the sampling effort involved, one day of biofilm growth was the shortest time point that could be feasibly investigated. At this point the bacteria had increased dramatically in numbers and the biofilm was still growing. After three days of growth the numbers of bacteria in the biofilm had peaked. The numbers then gradually declined and after six days the numbers were similar to those determined after one day before they decreased considerably by fourteen days. For convenience of sampling it was decided to sample at seven days rather than six days in subsequent experiments. The total viable counts are graphed against time in Figure 9.C.

This experiment was repeated five times (five cohorts of nine replicates for sampling periods of one, three and six days) to investigate reproducibility of results. (The fifth cohort was also investigated at nine and fourteen days to identify a suitable sampling point during the decline of the biofilm). The numbers of bacteria were statistically different between experiments (see Figure 9.C.ii showing 95% confidence intervals for the means of the different experiments, if the error bars do not overlap the means are statistically different). The patterns of biofilm growth (the shape of the curves) were similar between experiments.

To assess each time period of biofilm growth was process intensive so only up to about 12 biofilms were assayed on any one day. This is as total viable counts, viability Live/Dead counts, pH measurements and confocal pictures were taken. The time points, one day, three days, seven days and fourteen days were chosen to be used in subsequent experiments as they encompass the growth, maturation and decline of the biofilm.

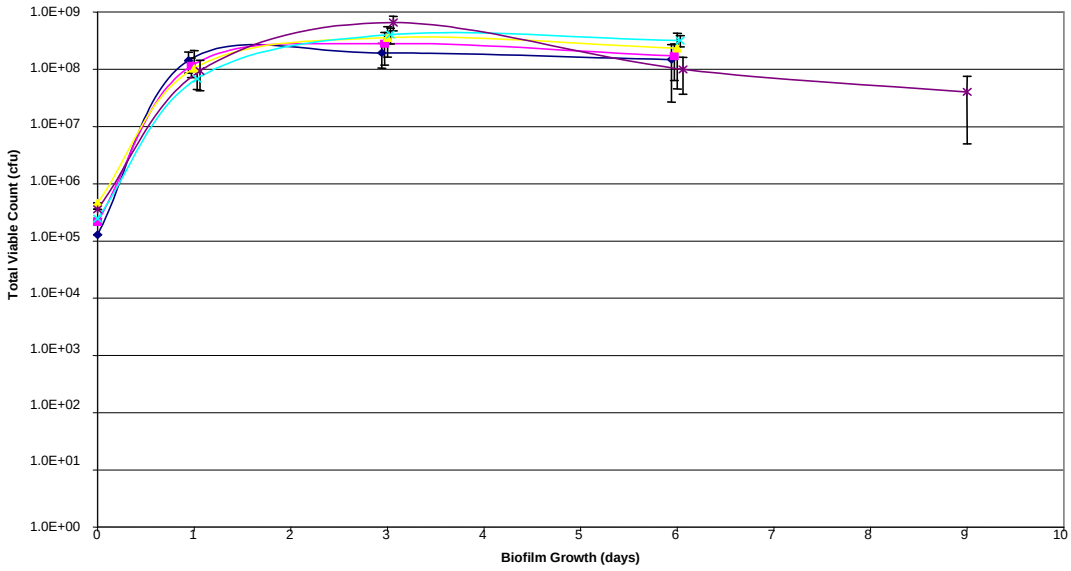


Figure 9.C. *S. mutans* biofilm growth over five independent experiments This study investigates reproducibility and suitable sampling points. This graph shows the *S. mutans* biofilms grew rapidly after inoculating the filters, with growth slowing after one day but continuing up to three days before beginning a decline in bacterial viable numbers after that. One cohort (purple) was also sampled at fourteen days where there were total viable counts of 2.5 x 10^4 cfu, 5.0 x 10^4 cfu and a further seven replicates with bacteria but less than 2.5 x 10^4 cfu (because of the greatly reduced numbers the correct plates in the serial dilution were not plated out but this preliminary study identified this for later experiments). The error bars show 95% confidence intervals for the means of the different experiments.

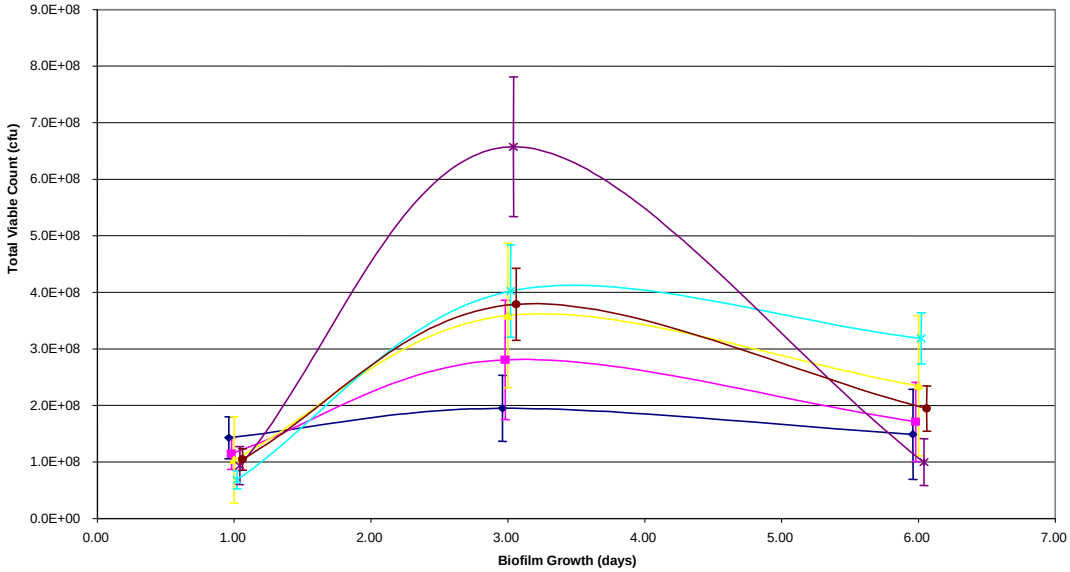


Figure 9.C.ii. *S. mutans* biofilm growth over five independent experiments showing 95% confidence intervals of the means (rather than standard deviations as used elsewhere) for each of the five experiments and for them all combined. This graph was not plotted on a logarithmic scale so the confidence intervals of the means can be more easily compared. This graph shows the similarity within cohorts and the differences between cohorts but also the similarity in trends between cohorts.

Figure 9.C.ii shows the inter-experimental variability and identified the need to carefully pair samples to investigate different treatments.

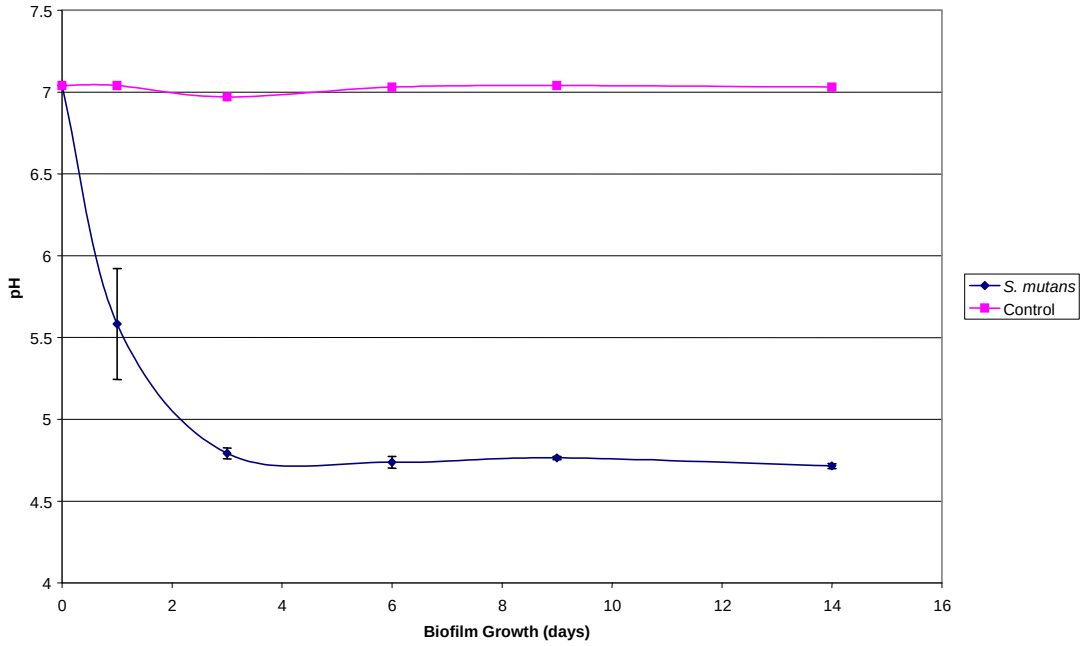


Figure 9.D. pH of *S. mutans* biofilms.
This figure shows the pH change over biofilm development in the fifth experiment with the means and standard deviations of the nine replicates of each time point shown. This figure shows the rapid decrease in pH of the biofilms.

This experiment was used as an opportunity to investigate different sampling methods including pH analysis, and viability analysis. These experiments determined the feasibility of different sampling procedures.

Figure 9.D shows the rapid increase in acidity in *S. mutans* biofilms and how the pH stabilises at about 4.75 after three days of biofilm growth. The small standard deviation bars show how consistent the pH reached in the biofilms was.

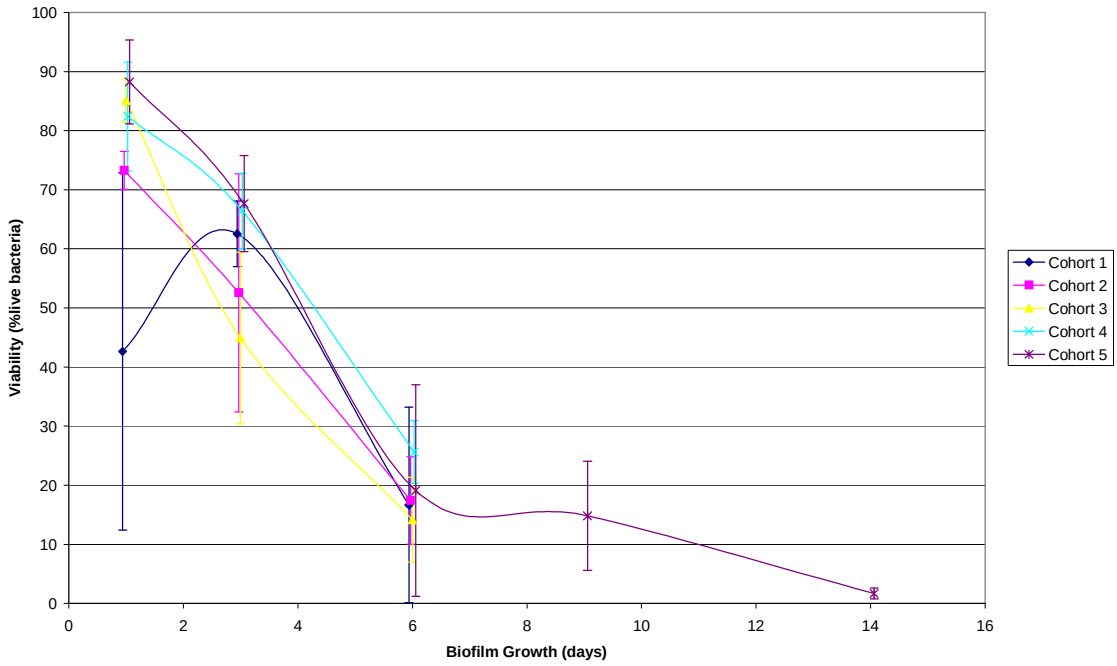


Figure 9.E. Viability of *S. mutans* biofilms. Five replicates of the fifth experiment were stained with LIVE/DEAD stain and counted to test the feasibility of the method.

Figure 9.E shows the viability of the bacteria within the biofilm. There were some live and dead bacteria within the biofilm at all time points. In the one day biofilms there was much variation in the vitality of biofilms. However, note the increase in averages of viability of day one biofilms in the later cohorts as the experimenter's proficiency and technique improved. After fourteen days of biofilm growth, the percentage of live bacteria in the biofilm was very low. This is consistent with the decline in total numbers of bacteria in the biofilm (see Figure 9.C). Note however that after six days, even though the majority of bacteria were dead, there were still approximately 10^8 bacteria in all biofilms.

9.2.2.3 Application of techniques to the growth and analysis of *V. dispar* biofilms

V. dispar biofilms were grown and analysed in the same way as *S. mutans* biofilms. *V. dispar* biofilms grew to similar numbers as *S. mutans* biofilms when sampled after one day of biofilm growth (*V. dispar* [1.48 x 10^8 ± 8.6 x 10^7 cfu] compared with *S. mutans* [1.05 x 10^8 ± 6.4 x 10^7 cfu], two tailed independent samples t-test, t=-1.734,

d.f.=51, p=0.089), (see Figure 9.F). *V. dispar* biofilms decreased in numbers after one day while *S. mutans* biofilms increased in numbers after one day and consequently three day biofilms of the two types differed significantly in numbers (*V. dispar* [1.27 x 10^8 ± 5.2 x 10^7 cfu] compared with *S. mutans* [3.79 x 10^8 ± 2.18 x 10^8 cfu], two tailed independent samples t-test, t=3.371, d.f.=51, p=0.001). After fourteen days of growth the *V. dispar* biofilms still had on average 1.39 x 10^6 ± 9.5 x 10^5 cfu bacteria, many more than *S. mutans* biofilms that had almost died out by this time (statistical test not done due to insufficient data). The biofilms display a similar growth, maturation and decline to the *S. mutans* biofilms except they decreased after one day but at a slower rate than the decrease in *S. mutans* biofilms. The medium under the biofilm increased in pH over time (see Figure 9.G). This is probably due to the breakdown of nitrate and nitrogenous bases which produce slightly alkaline end products. The increase in pH was more gradual than the decrease observed in *S. mutans* biofilms and on a smaller scale.

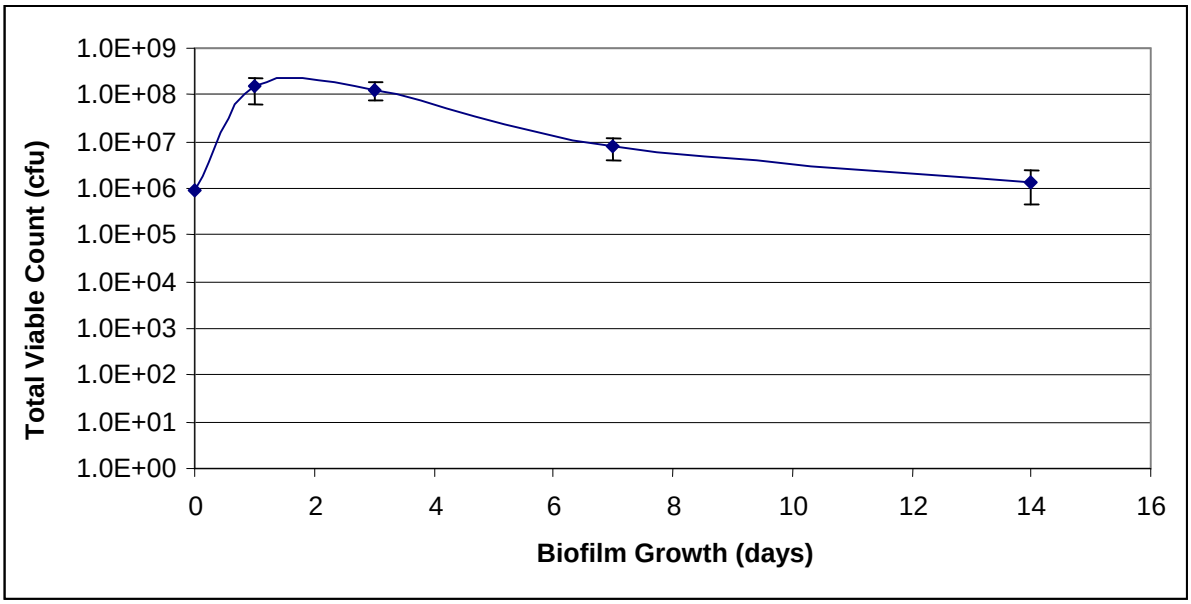


Figure 9.F. Total viable counts of *V. dispar* biofilms.

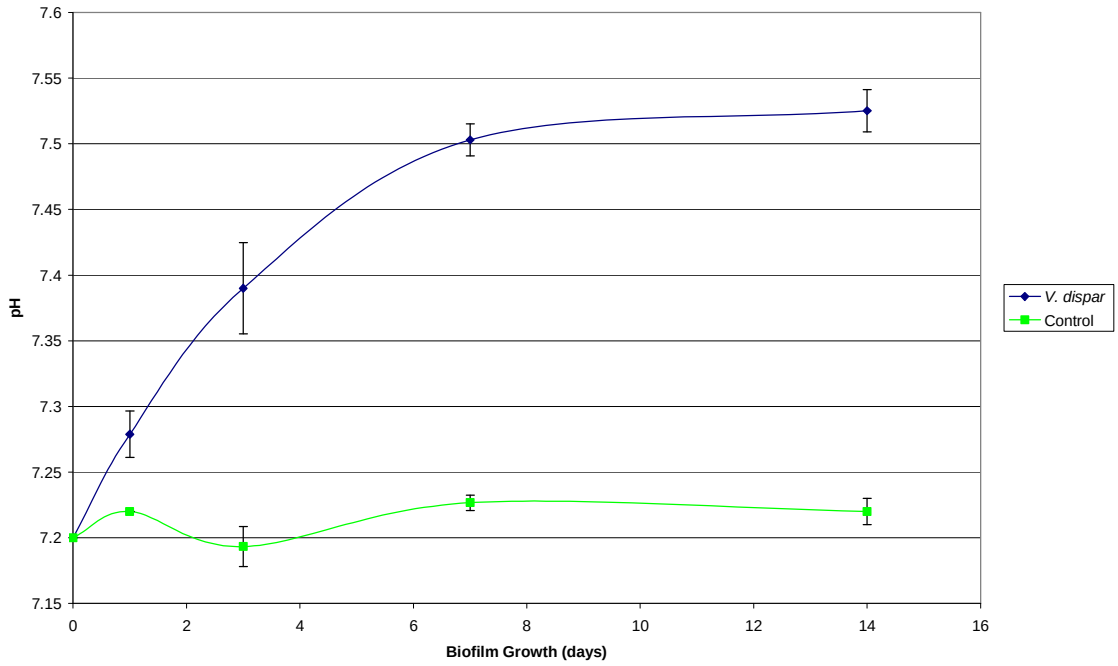


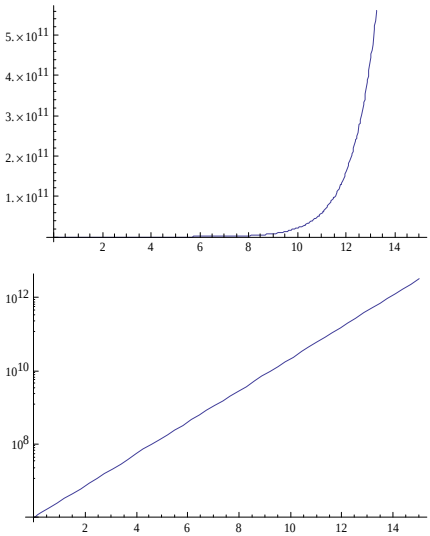
Figure 9.G. pH of *V. dispar* biofilms.

These preliminary studies determined the methods of growth and analysis for the subsequent experiments. To further allow comparison and reduce sampling error, each 6-well tissue culture plate was used to grow two *S. mutans* biofilms, two *S. mutans* and *V. dispar* biofilms and two *V. dispar* biofilms.

9.3 Appendix 2 – Mathematica Code

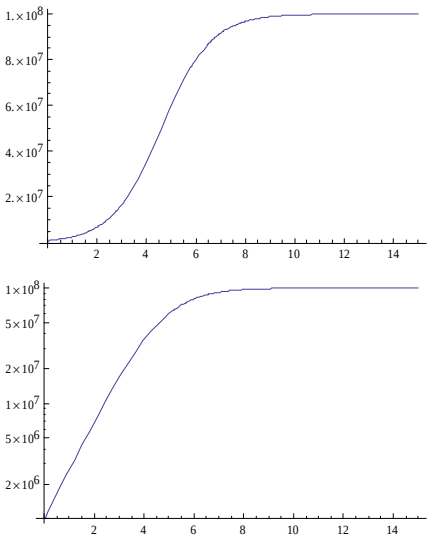
```
(* Exponential growth *)

ClearAll["Global`*"]
DSolve[{y'[x] == y[x], y[0] == a}, y, x] // Quiet;
Plot[Evaluate[y[x] /. % /. {{a → 1 000 000}}], {x, 0, 15}]
DSolve[{y'[x] == y[x], y[0] == b}, y, x] // Quiet;
LogPlot[Evaluate[y[x] /. % /. {{b → 1 000 000}}], {x, 0, 15}]
```



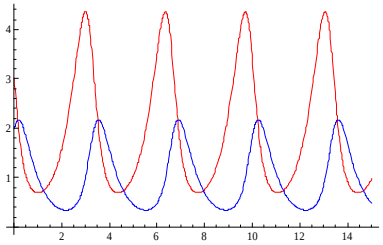

```
(* Logistic growth *)

ClearAll["Global`*"]
DSolve[{y'[x] == y[x] (1 - y[x] / 100 000 000), y[0] == a}, y, x] // Quiet;
Plot[Evaluate[y[x] /. % /. {{a → 1 000 000}}], {x, 0, 15}]
DSolve[{y'[x] == y[x] (1 - y[x] / 100 000 000), y[0] == b}, y, x] // Quiet;
LogPlot[Evaluate[y[x] /. % /. {{b → 1 000 000}}], {x, 0, 15}]
```



```
(* Lotka−Volterra predator prey equations *)

ClearAll["Global`*"]
LotkaVolterra[{a_, b_, c_, d_}, {x0_, y0_}, t1_] := Module[{x, y, t},
   {x, y} /. Quiet[NDSolve[{
        x'[t] == a x[t] - b x[t] y[t],
        y'[t] == -c y[t] + d x[t] y[t],
        x[0] == x0,
        y[0] == y0
       }, {x, y}, {t, 0, t1}]] // Flatten
  ];
a = 2; b = 2; c = 2; d = 1; prey0 = 3; pred0 = 2; tmax = 15; Module[
 {soln, col1 = RGBColor[1, 0, 0], col2 = RGBColor[0, 0, 1]},
 Plot[Evaluate[{#[[1]][t], #[[2]][t]} &[soln = Quiet@LotkaVolterra[{a, b, c, d}, {prey0, pred0}, tmax]]], {t, 0, Min[tmax, Min[#[[1, 1, 2]] & /@ soln]]},
  PlotRange → All, AxesOrigin → {0, 0}, PlotStyle → {col1, col2}, ImageSize → {400, 300}, ImagePadding → {{40, 10}, {10, 25}}]
]
```



```
(* Streptococcus mutans, Veillonella dispar, a competitor and lactic acid *)
(*
ds/dt = r s (1 - h/Φ),
dq/dt = w s (1 - h/y),
dv/dt = (α + ω h) v (1 - h/Ψ),
dh/dt = β s - φ v
*)
(*
s is the number of S.mutans,
v is the number of V.dispar,
q is the number of a competing species,
t is the time in days,
h is the concentration of lactic acid in mol/l,
Φ is the concentration of lactic acid in mol/l where growth of S.mutans ceases and beyond which is negative,
Ψ is the concentration of lactic acid in mol/l where growth of V.dispar ceases and beyond which is negative,
y is the concentration of lactic acid in mol/l where growth of a competitor ceases and beyond which is negative,
r is the growth rate of S.mutans, α is the growth rate of V.dispar in the absence of lactic acid,
ω is a factor that captures the increase in growth rate of V.dispar in the presence of lactic acid,
w is the growth rate of a competitor,
β is the amount of H+ produced by S.mutans,
φ is the amount of H+ consumed by V.dispar,
*)
ClearAll["Global`*"]
Koning[{Φ_, y_, Ψ_, r_, w_, α_, ω_, β_, φ_}, {s0_, q0_, v0_, l0_}, t1_] := Module[{s, q, v, h, t},
  {s, q, v, h} /. Quiet[NDSolve[{
      s'[t] == r s[t] * (1 - h[t]/Φ),
      q'[t] == w q[t] * (1 - h[t]/y),
      v'[t] == ω h[t] * v[t] + α * v[t] * (1 - h[t]/Ψ),
      h'[t] == β s[t] - φ v[t],
      s[0] == s0,
      q[0] == q0,
      v[0] == v0,
      h[0] == l0
     }, {s, q, v, h}, {t, 0, t1}]] // Flatten
 ];

Manipulate[
 Module[
  {soln, col1 = RGBColor[1, 0, 0], col2 = RGBColor[1, 0.5, 0], col3 = RGBColor[0, 0, 1], col4 = RGBColor[0, 1, 0]},
  LogPlot[Evaluate[{#[[1]][t], #[[2]][t], #[[3]][t], #[[4]][t]} &[
     soln = Quiet@Koning[{Φ, y, Ψ, r, w, α, ω, β, φ}, {S0, com0, V0, la0}, tmax]]],
   {t, 0, Min[tmax, Min[#[[1, 1, 2]] & /@ soln]]},
   AxesLabel → TraditionalForm /@ {t, {Style[S[t], col1], Style[com[t], col2], Style[V[t], col3], Style[la[t], col4]}},
   PlotRange → All, AxesOrigin → {0, 0}, PlotStyle → {col1, col2, col3, col4}, ImageSize → {400, 300},
   ImagePadding → {{40, 10}, {10, 25}}]
 ],
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 {{y, 0.000008, "y comp threshold"}, 0.00000000000001, 0.0001, ImageSize → Tiny, Appearance → "Labeled"},
 {{Ψ, 0.000005, "Ψ Vd threshold"}, 0.00000000000001, 0.0001, ImageSize → Tiny, Appearance → "Labeled"},
 {{r, 20, "r Sm g.r."}, 0.1, 200, ImageSize → Tiny, Appearance → "Labeled"},
 {{w, 25, "w Comp g.r."}, 0.1, 200, ImageSize → Tiny, Appearance → "Labeled"},
 {{α, 5, "α Vd g.r."}, 0.1, 200, ImageSize → Tiny, Appearance → "Labeled"},
 {{ω, 100 000, "ω Vd g.r. *"}, 0, 10 000 000, ImageSize → Tiny, Appearance → "Labeled"},
 {{β, 0.000000000000000000000001, "β L.A. prod by Sm"}, 0, 0.000000000000000000000001, ImageSize → Tiny,
  Appearance → "Labeled"}, {{φ, 0.000000000000000000000001, "φ L.A. cons by Vd"}, 0, 0.000000000000000000000001,
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 "initial values",
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 {{com0, 10 000 000, "Competitor"}, 100 000, 100 000 000 000 000 000, ImageSize → Tiny, Appearance → "Labeled"},
 {{V0, 10 000 000, "Veillonella"}, 100 000, 100 000 000 000 000 000, ImageSize → Tiny, Appearance → "Labeled"},
 {{la0, 0, "L.A."}, 0, 0.0002, ImageSize → Tiny, Appearance → "Labeled"}, Delimiter,
 {{tmax, 4, "maximum time"}, .01, 14, ImageSize → Tiny, Appearance → "Labeled"},
 SaveDefinitions → True, ControlPlacement → Left]
```
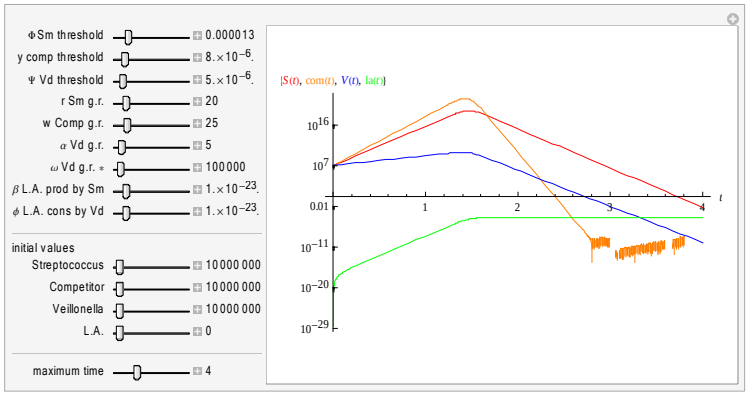
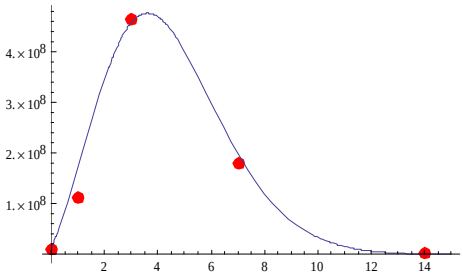
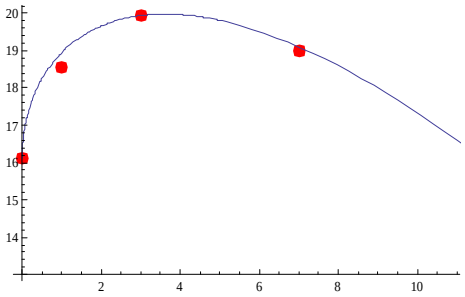

Out[15]=



```
(* Incipient growth processes with competing mechanisms *)

days = {0, 1, 3, 7, 14};
ss = {1.00 10^7, 1.13 10^8, 4.65 10^8, 1.81 10^8, 3.48 10^6};
coords = Table[{days[[k]], ss[[k]]}, {k, 1, 5}];
logcoords = Table[{days[[k]], Log[ss[[k]]]}, {k, 1, 5}];
a1 = ListPlot[coords, PlotStyle → {RGBColor[1, 0, 0], PointSize[0.03]}, DisplayFunction → Identity];
a2 = ListPlot[logcoords, PlotStyle → {RGBColor[1, 0, 0], PointSize[0.03]}, DisplayFunction → Identity];

y0 = 10^7; tc1 = 0.0435; m1 = 0.489; tc2 = 1.58; m2 = 1.37; compmech2 = y0 2^((t/tc1)^m1 - (t/tc2)^m2);

a3 = Plot[compmech2, {t, 0, 15}, DisplayFunction -> Identity];
a4 = Plot[Log[compmech2], {t, 0, 15}, DisplayFunction -> Identity];
Print["Binary logs"];
Show[a1, a3, DisplayFunction → $DisplayFunction]
Show[a2, a4, DisplayFunction → $DisplayFunction]

y0 = 10^7; tc1 = 0.09; m1 = 0.49; tc2 = 2; m2 = 1.35; compmechN = y0 Exp[(t/tc1)^m1 - (t/tc2)^m2];

a3 = Plot[compmechN, {t, 0, 15}, DisplayFunction -> Identity];
a4 = Plot[Log[compmechN], {t, 0, 15}, DisplayFunction -> Identity];
Print["Natural logs"];
Show[a1, a3, DisplayFunction → $DisplayFunction]
Show[a2, a4, DisplayFunction → $DisplayFunction]
```

Binary logs





Natural logs